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AN EVALUATION OF THE SPECIFICITY AND SENSITIVITY OF A GEL DOUBLE-DIFFUSION TEST FOR TUBERCULOSIS[1, 2]

A Double-blind Study

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Introduction

Earlier studies reported from this institution have shown that a gel diffusion technique is useful for the detection of antibodies against mycobacterial antigens. It was first applied to an investigation of the antigenic relationships between mycobacteria (1, 2). Subsequently, a preliminary study (3) indicated that it might be a sensitive method for the detection of antibody in the serum of patients suffering from tuberculosis.

Because of the limited number of patients involved in this initial study and because of the restricted nature of the patient population from which the serum samples were obtained, it was obvious that a much larger number of patients representing a greater diversity of disease entities must be tested before a valid indication could be obtained of the specificity of the test and of its usefulness as a diagnostic adjunct.

Therefore, on July 1, 1958, a cooperative study was initiated under the auspices of the Laboratory Subcommittee of the Committee on Medical Research of the American Trudeau Society. Physicians connected with 20 hospitals and sanatoriums in widely different parts of the United States agreed to send samples of serum from patients to the Department of Microbiology, Northwestern University Medical School, where they were to be tested by the gel double-diffusion technique for the presence of mycobacterial antibodies.

The primary aim of this double-blind study was to obtain objective data on the specificity of the gel diffusion test. For this reason the cooperative institutions were requested to send serum chiefly from patients suffering from diseases other than tuberculosis. It was hoped in this manner, not only to obtain information on specificity, but also to obtain information on the relationship between the occurrence of tuberculin hypersensitivity in clinically nontuberculous subjects and the presence or absence of mycobacterial antibodies in the serum of such people. In addition to the double-blind evaluation program using patients, studies were initiated on the effect of BCG vaccination on the appearance of antibodies in the serum of humans and on the effect of BCG vaccination and of tuberculous infection in monkeys on the appearance of mycobacterial antibodies.

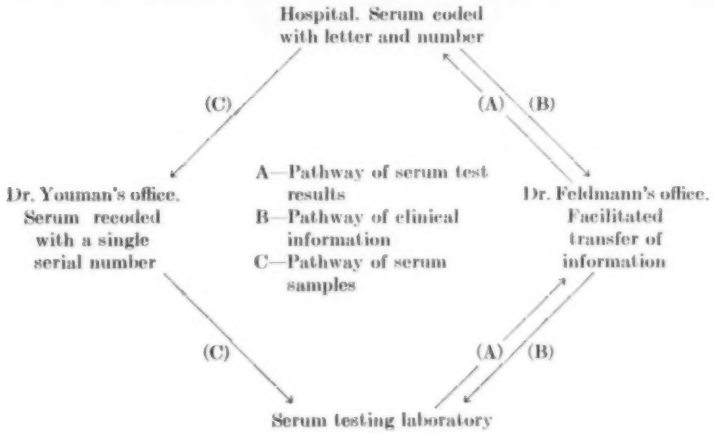
Finally, from the results of the tests on the sera from tuberculous patients, it was hoped that data would be available which might provide some indication of the usefulness of the test for the detection of tuberculous disease in humans.

The present paper will give the results of the cooperative study as they relate to the specificity of the gel diffusion test. In addition, data are given which will show the relationship between the state of tuberculin hypersensitivity and the presence of circulating antibody to mycobacterial antigens. Finally, some preliminary data are also given on the sensitivity of the test for the detection of antibody in the serum of tuberculous patients and on the detection of antibody in the serum of healthy males following BCG vaccination. Data on the immunologic response of monkeys will be given in a future publication.

[1] From the Department of Microbiology, Northwestern University Medical School, Chicago, Illinois.

[2] Supported by a grant from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association.

The sera and data were handled as indicated in the following diagram:



This procedure ensured that the people who performed the serologic tests would remain unaware of the type of patient from whom each serum sample was obtained and of the identity of the contributing institution.

The cooperating physicians were requested to forward directly to Dr. Floyd Feldmann, Director of Medical Research, American Trudeau Society, New York, the following data on each patient: patient's code number, name, age, sex, principal diagnosis, result of Mantoux test and type of testing material, nature of any coexisting disease, and comments on conditions which might affect immunologic response, such as stage of disease, pregnancy, and debilitation. The results of the gel diffusion tests on each serum specimen also were sent to Dr. Feldmann who effected the exchange of information between the testing laboratory and the clinical institutions.

Materials and Methods

Since earlier studies (1, 2) had demonstrated an apparent antigenic identity between the H37Ra and H37Rv strains of mycobacteria, the H37Ra strain was used for production of concentrated Proskauer and Beck culture filtrate antigens in the following manner.

Flash cultures of the Youmans modification of Proskauer and Beck medium (4) were inoculated from a pellicle culture of the H37Ra strain. These cultures were incubated at 37°C. for eight weeks. At the termination of incubation, the medium was separated from the cellular material by filtering twice through a medium-pore Berkefeld filter.

Antigens were concentrated by pervaporation by placing the filtrate in Visking tubes. The filled tubes were hung in front of an electric fan run at medium speed. The best results were achieved if 500-ml. amounts were placed in 2½- by 36-inch Visking tubing and pervaporated to 50 to 75 ml. in about thirty hours. Following concentration, the filtrates were removed from the tubing and filtered twice through fine-pore Berkefeld filters. If handled aseptically after the first of these filtrations, the material remained sterile following the second. Due to the bacteriostatic and protein protective effects of the concentrated residual glycerol in filtrates, this preparation was found to remain sterile and to be stable at room temperatures for several months.

As indicated previously, the antisera used in the actual evaluation study were supplied by various hospitals throughout the country. Considerable variation in the quality of the sera received was noted. The quality from specimen to specimen ranged from clear colorless sterile serum to very badly hemolyzed, lipemic, and/or contaminated sera. Regardless of the quality, however, all sera were set up in tests. In some cases, in which the above alterations of the serum prevented observation of the center column, it was necessary to discard tests after twenty-four hours of incubation. After the program had been underway for several months, it was found that false positive tests could be obtained with slightly hemolyzed, lipemic, or contaminated sera. This necessitated the discarding of the results on some previously tested samples of serum. Some other serum specimens were not tested in this evaluation because of breakage in transit or some other mechanical loss. Upon termination of the study it had been necessary to discard a total of 101 samples for the above reasons. This number included 56 sera from tuberculous patients, 10 sera from patients in whom the diagnosis was inconclusive, and 35 sera submitted from hospital

patients; 1,652 specimens of serum were actually used for the evaluation of the gel diffusion test.

The tubes[3] used for the gel diffusion test were identical to those described in an earlier report (3). They were made of soft glass tubing and were 10 cm. in length with an internal diameter of 3 mm. For filling the tubes, sterile disposable Wintrobe[3] capillary pipettes were used. All tubes and pipettes were discarded after each use.

The gel diffusion tubes were internally coated with a thin film of dried agar prior to performance of the tests. This coating was necessary in order to ensure adherence of the agar column to the wall of the tube. The coating was accomplished by inverting clean tubes in a beaker containing sufficient 0.1 per cent agar (prepared with distilled water) to cover the tubes. This beaker of tubes was then autoclaved at 15 pounds of pressure for fifteen minutes. While the agar was still fluid, the tubes were emptied of as much agar as possible by simply shaking the agar out of the tubes. The agar-coated tubes were then dried in a 50° C. hot-air oven for twelve hours, or overnight. Tubes were coated several days in advance of their use and stored in a dry place.

To perform the test an agar colum which contained antigen was placed in the coated tubes: A 2:1 ratio of antigen to agar mixture was prepared in a 50° C. water bath using melted 2 per cent Difco purified agar prepared with 1:100 M phosphate buffer at pH 7.5. Using a sterile, disposable Wintrobe capillary pipette, each tube was filled to a depth of 2.5 to 3.0 cm. A second 0.5-cm. deep layer of 1 per cent purified agar prepared with phosphate buffer was placed over the first antigen layer when it had gelled. Following solidification, undiluted serum was placed on top of the second layer with a sterile Wintrobe capillary pipette. The tubes then were placed in a clean container (a beaker or Mason jar), covered to protect from dust, and incubated at 37° C. The tests were observed at twenty-four-hour intervals.

It was found to be important at the time of the initial reading to moisten the outside of the tubes with water and polish them with a dry lint-free cloth.

It was also noted that visualization of the precipitate depended in part upon refraction of light by the precipitate. Therefore, a strong direct light which passed through the tubes obliquely greatly facilitated detection of fine precipitates.[4] Visualization was found to be easier if the overhead light in the room was dimmed and if the tubes were read against a dark background.

Readings were recorded each day for seven days according to the following key:

4-plus reaction—if 4 or more sharply defined dense bands of precipitate developed in the center reaction column.

3-plus reaction—if 3 bands of precipitate developed. These bands were not as well defined as those usually seen in the 4-plus reaction.

2-plus reaction—if 2 bands of precipitate appeared in the reaction tube. These appeared as separate bands but often lacked the sharp definition usually seen in 3- and 4-plus reactions.

1-plus reaction—if only one band of precipitate appeared. These were sometimes very sharply defined and at other times appeared as a more diffuse faint zone of precipitate.

Negative reactions—if no precipitation was seen in the center reaction column at any time during the five days of incubation.

[3] Manufactured by A. S. Aloe and Company, St. Louis, Missouri.

[4] Fluorescent desk lamp, model UL-P-2324-16, 118 volts, 60 cycles, 42 amperes, Dazor Manufacturing Company.

Results

For purpose of analysis of data, the patients from which the sera were obtained for the gel diffusion test were divided into the following ten groups:

Group 1: Patients with well established nontuberculous disease in whom there was no evidence of a coexisting tuberculous process.

Group 2: Diagnostic problems in which because of the nature of the disease process a diagnosis of tuberculosis could not be eliminated.

Group 3: Cases in which the clinical diagnosis was given as "far advanced pulmonary tuberculosis."

Group 4: Cases in which the diagnosis was given as "moderately advanced pulmonary tuberculosis."

Group 5: Patients with a clinical diagnosis of pulmonary tuberculosis but in whom the bacteriologic isolations yielded only atypical acid-fast bacilli. Because the majority of history cards failed to designate the type of atypical Myobacterium obtained, it was impossible to subdivide this group.

Group 6: Cases in which the clinical diagnosis was given as "minimal tuberculosis."

Group 7: Cases in which the clinical diagnosis was given only as "tuberculosis." No reference was given to the type

TABLE 1

RESULTS OF GEL DIFFUSION TESTS ON 466 CASES OF NONTUBERCULOUS DISEASE

Number of Patients	Number with Positive Mantoux Test	Clinical Diagnosis	Gel Diffusion Test Results	
			Positive	Negative
1	0	Viral myocarditis	0	1
1	0	Emphysema with cor pulmonale	0	1
1	1	Aortic valvular insufficiency of syphilitic etiology; hypertension	0	1
1	0	Angioneurotic edema	0	1
3	0	Essential hypertension	0	3
1	1	Aspiration pneumonia	0	1
2	0	Pulmonary embolus	0	2
1	0	Malignant melanoma	0	1
1	0	Carcinoma of the breast	0	1
1	1	Brain tumor, type unknown	0	1
1	1	Exogenous obesity and congestive heart failure	0	1
1	0	Epidemic typhus	0	1
1	1	Cushing's disease	0	1
1	0	Erysipelas	1	0
2	0	Typhoid fever	0	2
1	0	Hypoprothrombinemia	0	1
1	0	Idiopathic thrombocytopenic purpura	0	1
1	1	Cholelithiasis and hypertensive heart disease	0	1
1	0	Obstructive jaundice	0	1
1	0	Whipple's disease, diagnosed by laparotomy and biopsy	0	1
2	1	Multiple myeloma	0	2
2	1	Lymphomas	0	2
1	0	Lymphosarcoma	0	1
1	1	Acute myelogenous leukemia	0	1
1	0	Hodgkin's disease	0	1
12	7	Diabetes mellitus	0	12
1	0	Gastrocolic fistula	0	1
2	0	Diverticulosis of colon	1	1
1	1	Regional enteritis	0	1
2	1	Diarrhea and gastroenteritis	0	2
7	5	Hemorrhagic duodenal ulcers	0	7
1	0	Nephrosis	0	1
1	0	Wilson's disease	0	1
2	2	Nephrotic syndrome	0	2
1	0	Pyelonephritis and hydronephrosis	0	1
3	0	Heart disease—congenital	0	3
3	0	Heart disease—congestive failure	0	3
9	3	Heart disease—rheumatic	0	9
3	3	Heart disease—acute infarction	0	3
23	11	Heart disease—arteriosclerotic	0	23

TABLE 1—CONTINUED

Number of Patients	Number with Positive Mantoux Test	Clinical Diagnosis	Gel Diffusion Test Results	
			Positive	Negative
2	2	Anemias	0	2
1	1	Pseudomonas septicemia	0	1
1	1	Streptococcal septicemia	0	1
24	0	Brucellosis—titers of 1:160 to 1:640	1	23
1	0	Viral meningoencephalitis	0	1
1	0	Acute parotiditis	0	1
14	0	Mumps encephalitis—titers 1:4 to 1:64	0	14
6	0	Psittacosis encephalitis	0	6
1	0	Lymphogranuloma venereum	0	1
1	0	Western equine encephalitis	0	1
12	2	Sarcoidosis	0	12
39	0	Cataract, mainly senile	2	37
26	3	Syphilis	1	25
21	1	Psychologic and neurologic disorders	0	21
24	3	Dermatitis	0	24
1	0	Pemphigus vulgaris	0	1
1	0	Basal cell epithelioma	0	1
1	1	Psoriasis	0	1
1	1	Erythema induratum	0	1
1	1	Disseminated lupus erythematosus	0	1
1	1	Erythema nodosum	0	1
1	0	Radiation ulcer	0	1
1	0	Atopic eczema	0	1
1	0	Erythema multiforme	0	1
1	1	Ascites (laparotomy failed to reveal etiology)	0	1
5	1	Infectious hepatitis	0	5
5	3	Laennec's cirrhosis of liver	1	4
1	1	Fever of unknown origin, suspected lymphoma	0	1
1	0	Fever of unknown origin with nonspecific granulomatous liver lesion	0	1
1	0	Benign ulcer of the rectosigmoid colon	0	1
1	0	Acoustic neuroma	0	1
1	0	Thrombophlebitis	0	1
15	3	Asthma	0	15
3	3	Asthma with spontaneous pneumothorax	0	3
18	8	Pulmonary emphysema	0	18
2	2	Silicosis	0	2
3	1	Cystic disease of the lung	0	3
1	1	Nocardiosis, proved by culture	0	1
9	2	Chronic pulmonary histoplasmosis	0	9
1	1	Acute disseminated histoplasmosis	1	0
2	1	Disseminated coccidioidomycosis	0	2

TABLE 1—CONTINUED

Number of Patients	Number with Positive Mantoux Test	Clinical Diagnosis	Gel Diffusion Test Results	
			Positive	Negative
8	4	Nontuberculous bacterial pulmonary abscesses	0	8
16	11	Acute pneumococcal pneumonia	0	16
2	2	Acute staphylococcal pneumonia	0	2
10	6	Right-sided lobar pneumonia; etiology not specified	0	10
3	0	Viral pneumonia	0	3
1	1	Bronchopneumonia, *E. coli* isolated	0	1
1	1	Aspiration pneumonia	0	1
5	3	Left-sided lobar pneumonias; etiology not specified	0	5
37	23	Carcinoma of bronchi or lungs	1	36
12	6	Metastatic carcinoma of lungs	1	11
1	0	Thyrotoxicosis	0	1
2	0	Fractured rib and traumatic rupture of serratus anterior	0	2
1	0	Idiopathic headache	0	1
1	0	Fat embolus	0	1
1	1	Acute stomatitis	0	1
2	2	Chronic alcoholism	0	2
1	1	Dystonia muscularis deformans	0	1
1	1	Tetanus	0	1
1	0	Gonococcal cervicitis and arthritis	0	1
1	1	Thrombocytopemic purpura	0	1
1	0	Acute pancreatitis	0	1
1	1	Wernicke's syndrome	0	1
1	1	Multiple papillomas of the bladder	0	1
1	0	Polymyositis	0	1
466	152*		10†	456‡

* 32.6%
† 2.1%
‡ 97.9%

or degree of activity of tuberculosis nor was there any indication that the diagnosis had been confirmed bacteriologically.

Group 8: Cases in which it was stated that the tuberculosis was healed, arrested, or inactive. In the few cases in which the time between the last appearance of tuberculous activity and arrest or healing of the disease was given, it varied between one and twenty years.

Group 9: Cases stated to be extrapulmonary tuberculous disease. Because of the small number of cases given in this category, they have been grouped together, but they included: renal tuberculosis, osteotuberculosis, tuberculous peritonitis, tuberculous meningitis, miliary tuberculosis, and tuberculous lymphadenopathy.

Group 10: Cases of tuberculosis occurring in infants and children and stated to be "primary."

The results of the gel diffusion tests performed on the serum of 466 apparently nontuberculous persons (*Group 1*) are shown in table 1. One hundred and fifty-two (32.6 per cent) of the patients had some evidence of sensitivity to tuberculin. Four hundred and fifty-six specimens (97.9 per cent) of the serum of these patients were negative in the gel diffusion test. Only 10 (2.1 per cent) were positive. The 10 positive sera came from eight different disease entities and these included one case each of erysipelas, diverticulosis, brucellosis, syphilis, Laennec's cirrhosis, acute disseminated histoplasmosis, carcinoma of the bronchus, metastatic carcinoma of the lung, and 2 cases of senile cataract. The wide variety of disease entities from which these few positive sera were obtained strongly indicates that no true biologic false positive reactions occurred. Technical factors, or undiagnosed or healed tuberculous infections might have accounted for these positive reactions.

In table 2 have been listed the clinical data and gel diffusion test results on serum from 89 cases which were diagnostic problems, but in which tuberculosis as the etiologic factor had not been eliminated (*Group 2*). Fifty-six (62.9 per cent) patients had some evidence of tuberculin hypersensitivity. Twenty-four (27.0 per cent) had positive and 65 (73.0 per cent) had negative gel diffusion tests. Examination of table 2 will show that, in the main, sera with positive tests came from patients in whom there was a strong possibility that a tuberculous process might be involved.

The results of the gel diffusion tests on the sera of 1,097 patients stated to be tuberculous are set forth in table 3. The results indicate that in

TABLE 2
RESULTS OF GEL DIFFUSION TESTS ON 89 CASES IN WHICH TUBERCULOSIS CANNOT BE ELIMINATED

Number of Patients	Number with Positive Mantoux Test	Clinical Diagnosis	Gel Diffusion Test Results	
			Positive	Negative
4	4	Tuberculomas of the lung	2	2
1	1	Pulmonary granuloma; possible tuberculosis or coccidioidomycosis	1	0
4	4	Pulmonary disease of either tuberculous or deep mycotic etiology; agent not identified, possibly nocardia	0	4
1	1	Pulmonary abscess with several old small fibrotic areas elsewhere in the lung; etiology not determined	1	0
8	6	Chronic nonresolving pneumonias; etiologic agent undetermined	2	6
1	0	Generalized granulomatous lymphadenopathy; etiology remains undetermined	1	0
2	2	Pulmonary lesion of no established diagnosis; question of whether tuberculosis or malignancy	1	1
9	9	Pleural effusions of undetermined etiology	2	7
16	4	Bronchiectasis; unspecified etiology	1	15
3	1	Idiopathic hemoptysis	1	2
2	2	Hilar lymphadenopathy; undetermined etiology	0	2
23	14	Chronic inflammatory diseases of the lung with pulmonary fibrotic changes; cultures negative for acid-fast bacilli	6	17
1	0	Old calcific pleuritis, bilateral	0	1
1	0	Right middle lobe atelectasis	0	1
3	0	Middle lobe syndrome; etiology undetermined	3	0
1	1	Granuloma resected from lung; etiology remains undetermined; biopsy not conclusive; tuberculosis or sarcoidosis?	0	1
1	1	Infiltrate of right lung, not observed 7 months previously; culture negative for acid-fast bacilli	0	1
1	1	Right upper lobe cavity; cultures consistently negative for acid-fast bacilli	1	0
1	1	Agammaglobulinemia, bronchiectasis, and pulmonary fibrosis; cultures negative for acid-fast bacilli	0	1
1	1	Large superior mediastinal node; has remained unchanged for 12 months; observation for tuberculosis; cultures negative for acid-fast bacilli	1	0
1	0	Pyelonephritis with post-renal shutdown; azotemia with blood urea nitrogen of 44; etiology undetermined	1	0
1	1	Idiopathic pericarditis; biopsy negative for granulomatous change	0	1
1	1	Diagnostic problem: carcinoma of lung? carcinoma of pancreas? tuberculosis?	0	1
1	1	Chronic granulomatous infection of the neck; etiology undetermined	0	1
1	0	Respiratory neurosis; bilateral fibrocalcific pulmonary lesions; cortisone therapy	0	1
89	56 (62.9%)		24 (27.0%)	65 (73.0%)

pulmonary tuberculosis the farther advanced the disease process, the more likely the gel diffusion test is to be positive. This is suggested by the decline of the percentage of positive tests obtained from the far advanced cases of *Group 3* (84.2 per cent) to the minimal cases of *Group 6* (57.8 per cent). It is of interest to note that the percentage of positive sera from the patients suffering from atypical acid-fast *Group 5* infection (74.3 per cent) closely approximates the percentage of positive tests obtained from combining the data of *Groups 3*, *4*, and *6* (76.2 per cent).

However, there were not sufficient data available to permit a subdivision of the atypical cases on the basis of the degree of disease activity or on the basis of the type of atypical acid-fast bacilli, and it cannot be assumed that the distribution of type of disease was the same as for the typical cases. The results of the gel diffusion tests of serum from cases of atypical acid-fast infection does, however, confirm previous observations (2) which showed that the antigenic relationship between most atypical and typical strains of mycobacteria is very close.

TABLE 3

RESULTS OF GEL DIFFUSION TESTS OF 1,097 CASES OF TUBERCULOSIS

Groups	Number of Sera	Positive Mantoux Test	Gel Double Diffusion Test Reading				Total Tests		Per Cent of Tests	
			1+	2+	3+	4+	Positive	Negative	Positive	Negative
3	380	362	121	94	51	54	320	60	84.2	15.8
4	245	239	98	51	25	6	180	65	73.5	26.5
5	140	131	63	26	9	6	104	36	74.3	25.7
6	128	118	46	21	6	1	74	54	57.8	42.2
7	76	61	31	17	3	1	52	24	68.4	31.6
8	78	63	19	17	5	3	44	34	56.4	43.6
9	41	39	11	3	3	1	18	23	43.9	56.1
10	9	8	8	1	0	0	9	0	100.0	0.0
3, 4, 6	753	719	265	166	82	61	574	179	76.2	23.8

Group 7 comprises a collection of cases which were impossible to classify either as to the type of tuberculous infection or the degree of activity because of inadequate data. It is difficult to assess the significance of the 68.4 per cent of positive reactions which were obtained on the sera from these patients as compared with those obtained on the sera from the tuberculous patients in *Groups 3, 4,* and *6*.

Only a little more than 50 per cent of the arrested cases (*Group 8*) showed positive tests.

The gel diffusion test appears to be least effective for the detection of tuberculous infection in cases of extrapulmonary disease (*Group 9*). Only 43.9 per cent of the sera of such patients

TABLE 4

RESULTS OF GEL DIFFUSION TESTS ON 9 CASES OF PRIMARY TUBERCULOSIS

Case	Results of Mantoux Test	Test Results of Gel Double-Diffusion	Age	Clinical Data
			months	
1	6 mm. Battey Negative 5TU PPD	1+	10	History of contact with active case; reported Mantoux positive prior to admission; convalescent from measles
2	Negative Battey Negative PPD	1+	21	History of contact with active case; reported Mantoux positive prior to admission; convalescent from measles
3	15 mm. 5TU PPD Positive Battey Positive Bostrum	1+	36	History of contact with active tuberculosis; middle lobe syndrome
4	15 mm. 5TU PPD Positive Battey Positive Bostrum	1+	24	History of contact with active case; hilar lymphadenopathy
5	10 mm. 5TU PPD	1+	58	No history given
6	6 mm. 5TU PPD Positive Battey Positive Forbes	2+	24	History of contact with active tuberculosis; hilar lymphadenopathy
7	24 mm. 5TU PPD Positive Battey Positive Bostrum	1+	12	Middle lobe syndrome; gastric washing gave culture positive for tubercle bacilli
8	8 mm. 5TU PPD Positive Battey Positive Bostrum	1+	120	Progressive primary tuberculosis with typical cultures isolated
9	11 mm. 5TU PPD Positive Battey Positive Bostrum	1+	24	History of contact with father with active tuberculosis

TABLE 5

RESULTS OF GEL DIFFUSION TESTS ON 376 BACTERIOLOGICALLY PROVED CASES OF PULMONARY TUBERCULOSIS

Groups	Number of Sera	Positive Mantoux Test	Gel Double-Diffusion Test Readings				Total Tests		Per Cent of Tests	
			1+	2+	3+	4+	Positive	Negative	Positive	Negative
3	119	98	42	23	20	18	103	16	86.5	13.5
4	67	63	32	16	5	1	54	13	80.5	19.5
5	140	131	63	26	9	6	104	36	74.3	25.7
6	29	29	14	7	5	0	26	3	89.7	10.3
9	12	11	0	0	1	1	2	10	16.6	83.4
10	9	8	8	1	0	0	9	0	100.0	0.0
3, 4, 6	215	190	88	46	30	19	183	32	85.1	14.9

were positive. Whether this results from a disease process of short duration, from excess antigen produced by the widespread microorganisms, from immunologic paralysis, or from some unknown factor cannot be determined from these data. In view of the small number of cases in this category, a more extensive survey of the usefulness of this test for the detection of antibodies in such patients is needed.

Only 9 cases of primary tuberculosis in infants or children (*Group 10*) were available for study. Because of the fact that all 9 of these showed positive tests, all of the data concerned with these cases are presented in table 4.

In order to determine whether the use of more definitive diagnostic criteria would assist in the evaluation of the sensitivity of the gel diffusion tests for the detection of tuberculous disease, the data were reviewed, and only those cases were selected in which a definite statement had been made that tubercle bacilli had been isolated from the diseased individual. The results of the tests on the sera of these patients is shown in table 5. Comparison of the percentage values in table 5 with those in table 3 shows that by establishing this more rigid diagnostic criterion, the percentage of positive tests is increased. This is especially so in the minimal group cases (*Group 6*) in which the increase was from 57.8 to 89.7 per cent. The proportion of positive tests actually decreased, however, in the extrapulmonary cases (*Group 9*).

It is apparent from examination of the data in tables 1, 2, 3, 4, and 5 that there is no parallel between the status of tuberculin hypersensitivity of the patient and the results of the gel diffusion tests. The evidence is especially clear from the data in table 1 which gives results of tests on the sera obtained from the nontuberculous patients. A large number of these patients (32.6 per cent) had positive tuberculin tests but negative gel diffusion tests.

In order to test further whether the amount of antibody as measured by this technique was related to the degree of hypersensitivity, the analysis presented in table 6 was made using data from those patients with far advanced (*Group 3*), moderately advanced (*Group 4*), minimal (*Group 6*), and atypical (*Group 5*) pulmonary tuberculosis. Since there is a direct relationship between the amount of precipitate and the number of precipitate bands seen in the test and the amount of antibody in the serum (5), it is evident from the data in table 6 that there is no direct relationship between the amount of circulating antibody

TABLE 6

RELATIONSHIP BETWEEN MANTOUX TEST* READINGS, IN TERMS OF MM. OF INDURATION, AND GEL DOUBLE-DIFFUSION TEST READINGS IN 286 CASES OF BACTERIOLOGICALLY DIAGNOSED CASES OF PULMONARY TUBERCULOSIS

Class Sera	Gel Double-Diffusion Test Readings	Induration			
		5 mm.	6–10 mm.	11–20 mm.	>21 mm.
3 (89 cases)	0	1	4	7	0
	1+	1	9	23	2
	2+	0	5	12	0
	3+	2	5	6	0
	4+	0	3	6	3
4 (53 cases)	0	0	4	4	3
	1+	0	5	19	0
	2+	0	1	11	0
	3+	0	1	4	0
	4+	0	0	0	1
6 (28 cases)	0	0	1	5	5
	1+	0	2	6	2
	2+	0	0	2	2
	3+	0	0	3	0
	4+	0	0	0	0
5 (116 cases)	0	2	7	20	2
	1+	5	17	27	4
	2+	1	1	17	0
	3+	0	1	4	1
	4+	0	3	3	1

* 5 TU of PPD used in all skin tests.

and the degree of tuberculin hypersensitivity in these patients.

The results of a preliminary study in which 11 medical students were vaccinated with BCG by the multiple-puncture method showed that antibodies detectable by the gel diffusion test appeared in the sera of all subjects in eight to twelve weeks following vaccination. Antibody persisted in the sera of 2 students for sixteen weeks post vaccination, but was not detectable in any of the 11 students twenty weeks after vaccination.

Discussion

Since previous work (3) had indicated that the gel diffusion technique was reasonably sensitive, it seemed desirable to be certain of its specificity before extensive tests were done on the serum of tuberculous patients. The current investigation, therefore, was designed primarily to determine the specificity of this relatively simple technique for the detection of antibodies against mycobacterial antigens. This objective, however, was not achieved to the degree expected because most of the sera submitted for tests actually came from tuberculous rather than from nontuberculous patients. Also, 89 of the serum specimens submitted came from patients who constituted diagnostic problems (table 2).

The spectrum of nontuberculous pathologic conditions represented is wide, but the number of cases in many of the categories is too low to enable any conclusion to be drawn regarding the influence of many of the diseases on the gel diffusion test. However, the number of positive reactions found in the total number of nontuberculous patients examined was so small that strong presumptive evidence has been obtained which indicates that the specificity of the test is of a very high order. This high degree of specificity, as indicated by the small proportion (2.1 per cent) of false positive reactions, is in reality no more than one would expect from an antigen-antibody reaction of this type, since any direct precipitin test results in a direct visualization of the antigen-antibody complex.

One of the advantages of the gel diffusion precipitin technique is that it enables very small amounts of precipitated antigen-antibody complex to be seen. In all likelihood the majority of the 10 positive tests that were obtained in this group of nontuberculous cases actually resulted from the presence of antibodies against mycobacterial antigens present in the serum of these patients. On the other hand, a few of these positive reactions may have been artifacts due to nonspecific precipitation which may have resulted from the technical factors which will be discussed later in detail. It is unlikely, however, that the positive tests resulted from antibody engendered in these patients by the presence of some antigenically related substance. It would have been desirable, however, to have had more sera from patients suffering from acute and chronic infectious diseases in order to have adequately explored this point.

A serologic test to be of any real usefulness in the diagnosis of tuberculosis must not only be specific, but must be sensitive enough to pick up a significant number of cases of actual infection. On the other hand, such a test must not be so sensitive that it will detect those persons who have never suffered from tuberculosis but who have apparently acquired a tuberculous infection previously and as a result are tuberculin positive. With regard to this problem, the data provided by this study are clear. There is no significant positive correlation between the amount or presence of circulating antibody and the degree of hypersensitivity as determined by the Mantoux test. This is so for those patients suffering from nontuberculous infection and for those patients with active tuberculosis; it is also true for normal persons (3). This means that persons having positive Mantoux reactions need not have positive gel diffusion tests. On the other hand, it would be expected that persons who acquire tuberculous infection or disease and who become tuberculin hypersensitive may at the same time develop antibodies demonstrable by the gel diffusion test. That this does occur is shown by the data obtained on the gel diffusion test following BCG vaccination. Similarly whether antibody which would be detectable by the gel diffusion test might appear as a result of multiple intradermal injections of tuberculin also remains an open question. However, the data presented in this paper clearly show that the sera of the majority of Mantoux-positive clinically nontuberculous people do not give positive gel diffusion tests; this suggests the possibility that this serologic test may be more useful for the detection of actual cases of tuberculosis than the Mantoux test.

Although the major purpose of this study was not to determine the usefulness of the test for the detection of active tuberculosis in humans, most of the sera sent in actually came from tuberculous patients, and the results of the gel diffusion tests on these sera have been presented in tables 3, 4, and 6.

In an earlier publication (3) it has been pointed out by the present investigators that the results of serologic tests performed for the purpose of providing information on the presence or absence of an active infectious process are difficult to interpret. This becomes apparent if one considers that the detection of specific antibody by means of an antigen-antibody reaction may be considered as evidence for the presence of a currently active disease, a prior infection, prior vaccination, or the presence of a latent or inapparent infection. Furthermore, it was stated that frequently little reliance can be placed on the results of a serologic determination on a single serum sample. In order for serologic test results to have real significance, tests on several serial serum samples frequently are imperative. The necessity for this arises from the fact that the amount of specific antibody in the serum of a patient varies with individual ability to produce antibody in response to an antigenic stimulus, and to the effects on antibody levels in the serum of the continuous production of antigen by microorganisms in the tissues. In a chronic disease process such as tuberculosis, the latter may be of particular importance.

The information acquired in this study regarding the presence of antibody in tuberculous infection and disease indicates that tuberculosis is no exception and that the same factors regulate the presence of antibody in the serum as for other infectious diseases. For example, the results obtained from the study of vaccinated clinically well medical students are those that would be expected following exposure to small amounts of antigenic material. Without exception antibody appeared in these subjects several weeks subsequent to vaccination, persisted at relatively low levels for several more weeks, and then rapidly disappeared. On the other hand, there was indication that the sera from the 9 cases of primary tuberculosis had larger amounts of antibody in the serum. This could have resulted from the greater antigenization produced by clinical disease.

The fact that antibody may persist in the serum for a long period following recovery from tuberculous disease is illustrated by the fact that approximately 50 per cent of the patients with inactive or arrested disease (table 3) still had positive gel diffusion tests. Furthermore, one should bear in mind that nonspecific stimulation of antibody production possibly can occur in those persons who have previously been conditioned to produce specific antibody.

Finally in this connection, once antibody production has occurred, specific restimulation classically produces a more rapid and greater production of antibody which will gradually decrease to the point where it is undetectable, but will disappear only after a much longer period of time than that required following a single antigenic stimulus. Therefore, one would expect more antibody, and expect it to be present in the serum over a longer period of time, in cases of reinfection tuberculosis.

There is another factor, however, which undoubtedly serves to modify the above picture. This is derived from the fact that the amount of antigen produced by tubercle bacilli present in the tissues may be so great that the amount of antibody in the serum may be appreciably reduced, even to the point where it is undetectable. The situation may be a dynamic one in which the amount of antibody will decrease, or be absent, for the time the excess antigen is present, only to increase to a higher level following the disappearance of the antigen.

The data available from this study for the evaluation of the sensitivity of the gel diffusion test for the detection of tuberculous disease are inadequate because (except for 83 cases) only a single serum sample was available from each patient. For the reasons given in the preceding paragraph, some negative serologic tests in the face of active disease are to be expected. The question of primary importance is to what extent is the presence of detectable antibody in the serum of patients with tuberculosis actually affected by the amount, type, and location of the disease process. This question can be answered only by testing samples of serum obtained from patients at frequent intervals over a long period of time and then correlating these findings with the clinical condition of the patient. Such studies are now in progress.

It may be of interest, however, to report that in the 83 cases in which contributors submitted

more than one serum sample from each patient it was noted that in 36 an increase in the amount of antibody was observed in the second serum sample. Thirty showed no demonstrable change in amount of antibody in the second sample, and in 17 cases the antibody titer was lower on the second test than it had been on the initial test. In 26 of the cases the second serum sample was positive after the first serum sample had been negative. Of the 17 samples of serum which had shown a decrease in amount of antibody, 6 changed from positive to negative. The interval of time between the first and second serum samples in all cases was greater than four weeks, but in no case was it longer than twelve weeks. Although small in number, these data strongly support the value of repeated gel diffusion tests.

It follows, also, that, in order to evaluate the sensitivity of a serologic diagnostic test for the diagnosis of any disease, only cases should be employed in which the diagnosis is certain. For this reason only well-defined cases of tuberculosis in which the diagnosis has been substantiated by isolation of the positive organism should be used.

Even though this study did not meet all of the requirements for an adequate assessment of the sensitivity of this test, the results are believed to indicate that the gel diffusion test may hold considerable promise. The relatively high percentage of positive tests encountered in the various types of pulmonary infection indicates that this test may have considerable usefulness as a diagnostic tool. The work of Lester and Colton (6) also provides supporting evidence. These investigators found antibodies, detectable by the gel diffusion test, in the sera of about 94 per cent of their patients with bacteriologically diagnosed tuberculosis.

Investigations into the sources of error in the gel diffusion test itself led to the conclusion that certain conditions of the serum could result in error. Occasional specimens were encountered which rendered the center reaction column opaque following twenty-four hours of incubation. In all such cases it was noted that these sera were either hemolyzed, lipemic, or contaminated with bacteria. In a number of cases, second serum specimens obtained from the same patient, and which did not possess the quality noted in the first specimen, did not render the center column opaque. Certain factors apparently can bring about chemical changes, either in the agar itself or in impurities present in the agar. It is possible that one factor possessed in common by all of the above is an altered pH. This could be produced by fatty acids, steroid residues, by-products of bacterial metabolism, heme reduction or oxidations, et cetera. In those cases, however, in which the opacities were of lesser degree, it was sometimes difficult to differentiate them from weakly reactive antigen-antibody systems due to low antibody concentration in the serum. These chemically altered serum samples may account for some of the positive tests obtained with nontuberculous sera, especially since these "false positive" tests seldom exceeded a one-plus reaction. This source of error does not constitute a serious disadvantage in the gel diffusion reaction, however, as it is possible to eliminate almost completely such difficulties by specifying that fresh, sterile, nonhemolyzed sera from a fasting blood specimen be used.

Although the present study has revealed that the gel diffusion test for tuberculosis appears to possess a high order of specificity, and that the antibody levels in the circulation do not bear any close positive relationship to the status of tuberculin hypersensitivity of the individual, the following aspects of the test should be explored further before it is recommended for routine hospital use. A valid evaluation, carefully controlled, and with detailed follow-up study of cases when necessary, should be undertaken to determine more precisely the sensitivity of the test. The complete definition of the relationship to tuberculin hypersensitivity should be determined, and the relationship between positive serologic test results and BCG vaccination should be explored further.

Another problem encountered in the evaluation of the gel double diffusion test for tuberculosis was due to the difficulties found in standardizing antigen. Because of the experimental level of the current investigations, antigen was prepared in relatively small quantities. This meant that several batches of antigen were used throughout the evaluation study. A reference rabbit antiserum was maintained in the laboratory in order that a comparison of the effectiveness of antigen for precipitation of antibody might be made between two batches of antigen. As will be pointed out in a subsequent publication (5), this procedure is not entirely adequate.

Summary

A report is presented of the findings obtained from a large-scale double-blind evaluation of the usefulness of a gel double-diffusion test for the detection of antibody to mycobacterial antigens in the serum of human beings. The data were obtained using sera from 465 nontuberculous persons, 456 (97.9 per cent) of whom possessed no antibody to mycobacterial antigens by this test. One hundred and sixty-six (32.6 per cent) of the 465 persons showed some degree of tuberculin hypersensitivity. The question of the nature of the 10 (2.1 per cent) false-positive reactions is discussed. In 89 samples of serum from patients who were diagnostic problems and in whom tuberculosis had not been eliminated as a possible factor, it was found that 24 (27.0 per cent) gave positive gel diffusion tests for antibodies to tuberculosis.

Of 1,097 specimens of serum from tuberculous patients, it was found that in 380 cases of far advanced active pulmonary tuberculosis, 320 (84.2 per cent) of the gel diffusion tests were positive. In 245 cases of moderately advanced active pulmonary tuberculosis, 180 (73.5 per cent) sera were positive, while in 128 cases of minimally active pulmonary tuberculosis 74 (57.8 per cent) were positive. One hundred and forty sera from patients from whom only atypical acid-fast bacilli had been isolated were tested, and 104 (74.3 per cent) were found to contain mycobacterial antibody. In 78 sera from persons with either inactive tuberculosis or histories of "cured" tuberculosis, 44 (56.4 per cent) were positive. In 41 cases of extrapulmonary tuberculosis, 18 (43.9 per cent) were found to possess circulating antibody. All 9 cases of primary tuberculosis proved to be positive. In a miscellaneous group of 76 cases in which the clinical data were deficient in that neither activity, type of disease, nor laboratory data were given, 52 sera (68.4 per cent) proved to be positive.

Evidence is also given which indicates that the sensitivity of this test for detection of antibody to mycobacterial antigens appears greater if the test cases are limited to those in which the definite diagnosis of tuberculosis was made on the basis of bacteriologic isolation of atypical *M. tuberculosis*. Three hundred and seventy-six of the 1,097 cases of tuberculosis fell into this category. In the 119 sera from far advanced active pulmonary cases, the percentage of positive tests increased from 84.2 to 86.5 per cent. In 67 sera from moderately advanced cases, the increase was from 73.5 to 80.5 per cent. The 29 minimally active cases showed an increase of positive gel diffusion tests of 57.8 to 89.7 per cent.

There appeared to be no positive correlation between either the presence of tuberculin hypersensitivity and the presence of circulating antibody to tubercle bacilli, or, in those cases in which the gel diffusion tests were positive, between the size of the skin test and the amount of circulating antibody. It appears that the circulating antibody levels are independent of the allergic response to mycobacterial allergens.

Antibody was detected in the sera of 11 tuberculin-negative medical students only between eight and sixteen weeks after vaccination with BCG.

Sumario

Justipreciación de la Especificidad y la Sensibilidad de una Prueba de un Gel de Doble Difusión para la Tuberculosis: Estudio "Double Blind"

Preséntanse en esta comunicación los hallazgos obtenidos en una justipreciación "double blind" en gran escala de una prueba de un gel de doble difusión para el descubrimiento de anticuerpo de los antígenos micobacterianos en el suero de seres humanos. Se obtuvieron los datos usando sueros de 465 sujetos no tuberculosos, 456 (97.9 por ciento) de los cuales no poseían, conforme a esta prueba, ningún anticuerpo para los antígenos micobacterianos. Ciento sesenta y seis (32.6 por ciento) de las 465 personas revelaron en algún grado hipersensibilidad a la tuberculina. Se discute la cuestión de la naturaleza de las 10 (2.1 por ciento) reacciones seudopositivas observadas. En 89 ejemplares de suero de enfermos que constituían problemas de diagnóstico y en los que no se había eliminado la tuberculosis como posible factor, se observó que 24 (27.0 por ciento) acusaron pruebas positivas de gel-difusión para anticuerpos a la tuberculosis.

Entre 1,097 ejemplares de suero de tuberculosos, se observó que en 380 casos de tuberculosis pulmonar activa muy avanzada, 320 (84.2 por ciento) de las pruebas de gel-difusión fueron positivas. En 245 casos de tuberculosis pulmonar activa moderadamente avanzada, 180 (73.5 por ciento) ejemplares de suero resultaron positivos, mientras que en 128 casos de tuberculosis pulmonar mínimamente activa 74 (57.8 por ciento) fueron positivos. Se comprobaron 140 ejemplares de suero de enfermos de los que no se habían aislado más que bacilos ácidorresistentes atípicos, y en 104 (74.3

por ciento) se observó que contenían anticuerpo micobacteriano. De 78 ejemplares de suero de personas que tenían ya tuberculosis inactiva o historias de tuberculosis curada, 44 (56.4 por ciento) fueron positivos. Entre 41 casos de tuberculosis extrapulmonar, se observó que 18 (43.9 por ciento) poseían anticuerpo circulante. Todos los 9 casos de tuberculosis primaria resultaron ser positivos. En un grupo heterogéneo de 76 casos en los que los datos clínicos eran deficientes por no comprender la actividad y la forma de la enfermedad y en que no había datos de laboratorio, 52 ejemplares de suero (68.4 por ciento) resultaron positivos.

También se ofrecen datos indicativos de que la sensibilidad de esta prueba para el descubrimiento de anticuerpo a los antígenos micobacterianos parece mayor si se limitan los casos ensayados a aquéllos en que se hizo el diagnóstico positivo de tuberculosis a base del aislamiento bacteriológico de *M. tuberculosis* atípicas. Trescientos setenta y seis de los 1,097 casos de tuberculosis correspondieron a esta categoría. En los 119 ejemplares de suero de casos pulmonares activos muy avanzados, el porcentaje de pruebas positivas aumentó de 84.2 a 86.5. En 67 ejemplares de suero de casos moderadamente avanzados, el aumento fué de 73.5 a 80.5 por ciento. Los 29 casos mínimamente activos mostraron un aumento en pruebas de gel-difusión positivas de 57.8 a 89.7 por ciento.

Agréguese que no pareció que hubiera correlación positiva entre ya la presencia de hipersensibilidad a la tuberculina y la presencia de anticuerpos circulantes a los bacilos tuberculosos o, en los casos en que las pruebas de gel-difusión fueron positivas, entre el tamaño de la cutirreacción y la cantidad de anticuerpo circulante. Parece que las cifras de anticuerpos en la circulación son independientes de la respuesta alérgica a los alergenos micobacterianos.

No se descubrió anticuerpo en los sueros de 11 estudiantes de medicina, tuberculino-negativos, sino de ocho a dieciséis semanas después de la vacunación con BCG.

Resume

Evaluation de la spécificité et de la sensibilité du test de double-diffusion dans le gel dans la tuberculose: Une étude "double-blind"

Présentation d'un rapport sur les résultats recueillis à la suite d'une évaluation "double-blind" effectuée à grande échelle à l'égard de l'utilité du test de double-diffusion dans le gel, pour rechercher dans le sang humain les anticorps aux antigènes mycobactériens. Les données furent obtenues à l'aide de sérums provenant de 465 sujets non-tuberculeux, chez 456 (97,9%) d'entre eux, le test démontrait l'absence d'anticorps aux antigènes mycobactériens. Parmi ces 465 sujets, 166 (32,6%) présentaient un certain degré d'hypersensibilité à la tuberculine. Le problème de la nature des 10 (2,1%) réactions faussement positives est étudié. Dans les 89 spécimens de sérum provenant de malades posant un problème diagnostique et chez lesquels la tuberculose n'a pas été éliminée comme étant un facteur possible, 24 (27%) donnaient des tests de diffusion dans le gel positifs pour les anticorps à la tuberculose.

Sur 1097 spécimens de sérum provenant de tuberculeux, il a été découvert que dans 380 cas de tuberculose pulmonaire active très avancée, 320 (84,2%) tests de diffusion dans le gel étaient positifs. Dans 245 cas de tuberculose pulmonaire active modérément avancée, 180 (73,5%) spécimens de sérum étaient positifs, alors que dans 128 cas de tuberculose pulmonaire active minimale, 74 (57,8%) étaient positifs. Cent-quarante spécimens de sérum provenant de malades, chez lesquels on n'avait isolé que des bacilles acido-résistants atypiques, ont été testés et 104 (74,3%) contenaient l'anticorps mycobactérien. Dans 78 spécimens de sérum provenant de sujets présentant une tuberculose inactive ou des antécédents de tuberculose guérie, 44 (56,4%) étaient positifs. Dans 41 cas de tuberculose extrapulmonaire, 18 (43,9%) présentaient des anticorps circulants. Tous les 9 cas de tuberculose primaire se sont montrés positifs. Dans un groupe mixte comportant 76 cas pour lesquels les données cliniques étaient déficientes, en ce sens qu'on ne disposait pas de données sur l'activité, le type de maladie, ou de renseignements de laboratoire, 52 spécimens de sérum (68,4%) se sont montrés positifs.

Les auteurs fournissent également d'autres données indiquant que la sensibilité de ce test pour la détection d'anticorps aux antigènes mycobactériens semble plus grande si les cas testés ne comprennent que ceux dans lesquels un diagnostic de tuberculose avérée a été porté sur la base de l'isolement bactériologique de *M. tuberculosis* atypique. Trois-cent-soixante-seize cas sur les 1097 tombent dans cette catégorie. Dans les 119 spécimens de sérum provenant de cas de tuberculose active très avancée, le pourcentage de tests positifs augmentait de 84,2 à 86,5%. Dans les 67 specimens de sérum des cas modérement avancés, l'augmentation était de 73,5 à 80,5%. Les 29 cas de tuberculose active minimale montraient un accroissement de 57,8 à 89,7% du test de diffusion en gel positif.

En outre, on n'a décelé aucune corrélation positive entre la présence d'une hypersensibilité à la tuberculine et la présence d'anticorps circulants

aux bacilles tuberculeux ou, dans les cas où les tests de diffusion dans le gel étaient positifs, entre l'importance de la réaction cutanée et la quantité d'anticorps circulants. Il apparait que la concentration des anticorps circulants est indépendante de la réponse allergique aux allergènes mycobactériens.

Ce n'est que huit à seize semaines après vaccination au BCG que les anticorps ont été décelés dans le sérum de 11 étudiants en médecine.

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CLINICAL AND IMMUNOLOGIC SIGNIFICANCE OF ASPERGILLUS FUMIGATUS IN THE SPUTUM[1]

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INTRODUCTION

The importance of air-borne fungal spores as allergens in man is being increasingly recognized. Of the fungi, the Aspergillus genus, and in particular *A. fumigatus*, was first reported by V. Leeuwen (1) and subsequently by others, including Hansen (2), as perhaps the most important fungal cause of respiratory tract allergic disorders in their respective countries. In more recent years the Aspergillus genus has been ranked high in the list of inhaled fungal spore allergens by Feinberg (3) and Maunsell (4).

Aspergillus fumigatus may, however, produce infections in man, animals, and birds. In their report on pulmonary infection in man, entitled bronchopulmonary aspergillosis, Hinson and others (5) described incidents of transient lung shadows with eosinophilia in the peripheral blood, which they attributed to hypersensitivity, although limited skin testing did not provide evidence for this.

The spores of *A. fumigatus* like those of the other Aspergillus species are ubiquitous in their distribution and are present perennially. They are probably frequently inhaled and expectorated by the population at large, so that culture of *A. fumigatus* from the sputum cannot, by itself, be taken as proof of a causal relationship to the clinical manifestations.

The purpose of this investigation is to assess the possible relationship of the Aspergillus species to various respiratory disorders.

CLINICAL ASPECTS

MATERIALS AND METHODS

Clinical Material

The sputa of 2,080 patients with various chest disorders who attended the Brompton Hospital were cultured for *A. fumigatus*. Cultures positive for *A. fumigatus* were obtained in 145 patients, that is, about 7 per cent of the total. Of these patients, 1,060 were the subject of a previous mycologic study (6). The records of these 145 patients were examined, and relevant data were abstracted. Twenty-seven of the patients were admitted to the hospital for special study, including investigation of bronchial hypersensitivity.

The patients were questioned for evidence of personal or familial allergic disorders. The sputum and peripheral blood were examined for eosinophils, and repeated sputum cultures were made in the subjects studied in detail. Skin tests were performed in all patients with Aspergillus and other allergenic extracts, including grass, flower, and tree pollens, house-dust, and the fungi Cladosporium, Alternaria, Penicillium, Botrytis, Merulius, and Candida.[2] In 5 patients bronchographic and bronchoscopic examinations were made, and in 8 patients the maxillary antra were washed out and the washings cultured.

Direct bronchial sensitivity tests: Bronchial hypersensitivity was determined by giving inhalations for sixty seconds of nebulized mists of carbol-saline (Coca's fluid) extracts of Aspergillus to all of the patients and of other appropriate allergens to 12 patients. Repeated measurement of the forced expiratory volume in one second was made on a spirometer over the next fifteen minutes (7, 8). A consistent fall in the forced expiratory volume in one second greater than any fall that may have occurred following the control inhalation of Coca's fluid was regarded as evidence of bronchial hypersensitivity.

Method of sputum culture: Sputum samples were homogenized with pancreatin and the homogenate was inoculated liberally onto Sabouraud dextrose agar plates, which were incubated at 37°C. Colonies of *A. fumigatus*, if present, usually appeared within forty-eight hours, but plates were kept for seven days before discarding. Sporing cultures of *A. fumigatus* are typically smoky-green in color and show, on microscopic examination, flask-shaped vesicles, typically fertile over the upper half with the sterigmata crowded together in one series.

RESULTS

Prevalence of A. fumigatus and other Aspergillus species in the sputum in respiratory disorders: A. fumigatus was cultured from the sputum of 145

[1] From the Allergy Department, Institute of Diseases of the Chest, and the Bacteriology Department, Brompton Hospital, London, England; the Physiology Department, Chelsea College, London, England.

[2] Made by Duncan-Flockhart, Edinburgh, Scotland.

(7 per cent) of the 2,080 patients examined. The diagnoses of the respiratory disorders by the clinicians were as follows: asthma, 42 cases; asthma with pulmonary eosinophilia, 17; pulmonary eosinophilia without asthma, 4; mycetoma, 4; bronchitis, 37; bronchiectasis, 9; tuberculosis, 9; pneumonia, 7; bronchial carcinoma, 6; lung abscess, 3; sarcoidosis, 2 cases; other disorders, 5.

The incidence of *A. fumigatus* in the sputum of asthmatic and nonasthmatic subjects is given in table 1. In order to exclude any bias arising from more intensive search for the fungus in the sputum of asthmatics or subjects with pulmonary eosinophilia, the frequency with which the fungus was found in the first culture of sputum in the two groups was examined. The "positive" cases were then excluded, and the numbers with positive cultures on the second examination, the first being negative, were compared, and so on up to 7 sputum cultures. Thus, there were 8.3 per cent of patients in the asthmatic group with culture positive for *A. fumigatus* in the first sputum specimen examined, as compared with 3.4 per cent of patients in the nonasthmatic group. For those whose second examination of sputum was positive for the fungus the figures were 7.8 per cent for the asthmatic and 1.5 per cent for the nonasthmatic. These findings show a statistically highly significant ($P < 0.001$) greater frequency of the fungus in the sputum of asthmatics. The numbers available for comparison after culture of the third sputum specimen were too small for calculation of statistical significance.

In 23 (1.2 per cent) of the 2,080 patients, other Aspergillus species were cultured from the sputum. In 9 of these patients, the same or different specimens of sputum yielded *A. fumigatus* as well, and in several of these patients a third species was also found. The numbers of cultures were, *A. niger*, 11, *A. nidulans*, 5, *A. terreus*, 4, *A. flavus*, 2, *A. candidus*, 2, and *A. versicolor*, 1. In 4 of the 11 patients with *A. niger*, *A. fumigatus* was found, and in one of these *A. flavus* was also found. In 4 of the 5 patients with *A. nidulans*, one had *A. fumigatus* and *A. terreus* as well. In 2 of the 4 patients with *A. terreus*, *A. fumigatus* was found, and in one of these 2, *A. nidulans* was also found. In one of the 2 patients with *A. flavus*, *A. fumigatus* and *A. niger* were found. Of the 9 patients with *A. fumigatus* also present in the sputum, 7 were diagnosed as having asthma and 2 as having bronchitis. Of the remaining 14 subjects with one Aspergillus species cultured from the sputum other than *A. fumigatus*, 4 were diagnosed as having asthma and 10 as having bronchitis; 7, bronchiectasis and emphysema; 2, pulmonary tuberculosis; and one, carcinoma of the bronchus. These findings indicate that *A. fumigatus* rather than the other Aspergillus species was linked to the asthma.

TABLE 1

INCIDENCE OF SPUTUM CULTURE POSITIVE FOR A. FUMIGATUS

Order of Sputum Specimens Examined from Each Patient	Patients with Asthma			Bronchitis, Bronchiectasis, and Pneumonia		
	Number of Specimens	Positive		Number of Specimens	Positive	
		Number	Per Cent		Number	Per Cent
First	315	26	8.3*	1,000	34	3.4
Second (negative on first)	167	13	7.8	402	6	1.5
Third (negative on first and second)	89	5	5.6	150	4	2.7
Fourth (negative, first to third)	54	4	7.4	61	2	3.3
Fifth (negative, first to fourth)	20	3	15.0	17	3	17.6
Sixth (negative, first to fifth)	18	1	12.0	0	—	—
Seventh (negative first to sixth)	7	2		0	—	—

* *A. fumigatus* was cultured with a statistically significant, greater frequency ($P < 0.001$), from the first and second sputum specimens (percentages underlined) of asthmatics than from nonasthmatics.

Results of bronchial hypersensitivity tests: In order to study the significance of *A. fumigatus* in the sputum, 27 patients, 23 of whom were asthmatic, were especially investigated by direct bronchial sensitivity tests.

Aspergillus carbol-saline extract in concentrations of 1 or 10 per cent weight/volume (w/v) was used for the inhalation, the initial test concentration being influenced by the clinical condition and the skin test reactions. A positive reaction consisted of a fall in the forced expiratory volume in one second with or without asthma, depending upon the severity of the reaction. These symptoms were rapidly relieved by inhalation of isoprenaline. In some cases wheezing

TABLE 2

DIRECT BRONCHIAL TEST REACTIONS TO ASPERGILLUS EXTRACT
Clinical Features in 27 Patients with *A. fumigatus* in the Sputum

	Positive Bronchial Test Reactors Group 1 (16)	Negative Bronchial Test Reactors Group 2 (11)
Age and sex	12–61 years. Males 8, Females 8	13–67 years. Males 6, Females 5
Family history of allergy	8/16	4/11
Personal history of allergy	7/16 Eczema, hay fever, allergic rhinitis	4/11
Asthma	15/16 Usually from childhood, recently worse; recent onset in middle life in 3 cases	9/11 Usually from childhood; recent onset in middle life in 3 cases
Recurrent febrile episodes	15/16	0/11
Transient roentgenographic shadows	13/16 Often segmental collapse-consolidation	0/11
Sputum	6/16 Firm plugs; often mucopurulent and scanty	No plugs
Sputum eosinophilia	13/15	9/10 (1—not available)
Blood eosinophilia	15/16 More than 1,000 cu. mm. 9/16	6/11 More than 1,000 cu. mm. 2/11
Bronchiectasis (bronchogram)	5/5 At site of previous shadows	0/2
Skin sensitivity	16/16 Positive reactions to Aspergillus extracts; other allergens—average 4 positive per patient	2/11 Positive reactions to Aspergillus extracts; other allergens—average 1 positive per patient

recurred later and lasted for hours or days. A late reaction coming on about six hours later, in the absence of an immediate reaction, also occurred in one patient. One patient not only developed an asthmatic reaction, but also a recurrence of fever, diffuse pulmonary shadows, and blood eosinophilia.

Of the 27 patients, 16 (*Group 1*) gave positive bronchial reactions to Aspergillus extract, while the remaining 11 (*Group 2*) did not react. The clinical features of these two groups are given in table 2.

Clinical features in 16 patients with bronchial hypersensitivity (*Group 1*, table 2): In 8 of the 16 patients there was a family history of allergy. In 14 patients asthma was the presenting symptom, and in 7 of them other allergic disorders were also present. The asthma was of long standing in 11 patients and had become more severe in recent months, changing on occasion from seasonal to perennial asthma. In 3 patients the asthma was of recent onset, six months or less, and started in middle life. These latter cases resemble those of Hinson and his colleagues (5), in whom long-standing asthma was not a feature.

Roentgenographic evidence of transient pulmonary shadows, clearing within a few days or weeks, was available in 13 of the 16 patients. The shadows were usually segmental, having the appearance of consolidation–collapse, although they could also be patchy and nonsegmental. While these manifestations could be quite symptomless, they were sometimes associated with febrile episodes, which were present in 15 of the 16 patients. The sputum tended to be mucopurulent and scanty, and commonly contained eosinophils in large numbers. Tenacious plugs were expectorated by 6 patients like those noted by Hinson and others (5) in their cases. The plugs consisted of eosinophils and debris and were usually found during the febrile episodes. Fungal mycelium could be demonstrated in the plugs by silver impregnation and fibrin stains.

Blood eosinophilic counts of more than 500 per cu. mm. were found in 15 patients, the count in 9 of these being more than 1,000 per cu. mm. Raised eosinophilic counts were not consistently present.

In the 5 patients on whom bronchographic examination was performed, abnormally dilated bronchi were found, and appeared to develop at the sites of the previous transient shadows. This suggested that the dilatation may have been due to localized plugging or overdistension of the bronchi. Bronchoscopy was performed, and in 4 of the 5 patients excessively tenacious mucus plugging the bronchus was found. The bronchial

mucosa was edematous and excessively red in 3 of the patients.

On prick testing of the skin, all 16 of the patients gave positive reactions to Aspergillus extracts, and 15 gave positive reactions to other test allergens as well.

In the 2 subjects in this group without a history of asthma but with pulmonary eosinophilia and *A. fumigatus* in the sputum, both skin and bronchial tests nevertheless gave positive reactions.

Bronchial tests were performed in 6 patients in this group with other allergens to which positive skin test reactions were obtained. In 4 of the 6, bronchial reactions were elicited with Aspergillus extract only, while the remaining 2 reacted to two other allergens as well in each case.

Clinical features in the 11 patients without bronchial hypersensitivity (Group 2, table 2*):* The patients of *Group 2* resembled those of *Group 1* in the frequency of a family history of allergy and a personal history of asthma and other allergic disorders, and in the appearance of sputum and blood eosinophilia. They differed from the *Group 1* patients in certain important respects. None of them had recurrent pyrexial episodes, transient lung shadows, or sputum plugs. Only 2 gave positive reactions to skin tests with Aspergillus extracts and 4 of them reacted to other test allergens.

No reactions were elicited by bronchial tests with other allergens in 3 patients, only one of whom gave any positive skin test reactions to them.

Pulmonary Aspergillus mycetomata: In 4 patients Aspergillus mycetomata were found in the lungs. They were characteristic roentgenographically and appeared as a central solid body surrounded by a halo of air. This was well demonstrated by tomography.

It was possible to obtain further information in 3 patients. In 2 of these the fungus grew in a preformed air space: one in a pyogenic lung abscess and the other in an apical air cyst in lung damaged by fibrotic sarcoidosis; and in the third case, in a segment with a partially narrowed bronchus. The formation of mycetomata seemed to depend upon the existence of previous bronchopulmonary disease and did not appear to be related to hypersensitivity to Aspergillus. One of these 3 patients, in addition to having a mycetoma, had a long history of asthma. This patient showed bronchial hypersensitivity to Aspergillus extracts, and had eosinophilia, febrile episodes, and multiple fleeting pulmonary shadows seen roentgenographically, presenting a typical picture of allergic pulmonary aspergillosis with pulmonary eosinophilia. The other 2 patients were not asthmatic, had none of the features of pulmonary eosinophilia, and had no bronchial hypersensitivity to Aspergillus extracts.

Examination of the upper respiratory tract: In 8 patients with roentgenographic opacity of the antra, the maxillary antra were washed out, and in 2 of these *A. fumigatus* was cultured from the washings. One patient blew brown plugs from the nose, resembling those found in the sputum. These brown plugs also contained *A. fumigatus.*

Immunologic Investigations

In addition to the bronchial sensitivity tests, the immunologic investigations have consisted of:

Comparison of various extracts of a number of Aspergillus species by:

Skin tests in man

Serologic tests with rabbit antisera

Electrophoretic investigation of antigenic and allergenic components of *A. fumigatus.*

Serologic tests with human sera and Aspergillus extracts.

MATERIALS AND METHODS

Skin Tests

Six members of the Aspergillus genus, *A. fumigatus, A. flavus, A. glaucus, A. nidulans, A. niger,* and *A. terreus,* were cultured on Sabouraud peptone-glucose broth. Three types of extract were prepared: Carbol-saline (Coca's fluid) extract of dried defatted mycelium (10 per cent w/v), culture medium filtrate derived from one-month-old cultures after removal of the mycelium and Seitz filtration; cell-sap derived from washed mycelial mats of one-month-old cultures. The washed mats were mashed to a pulp, the fluid portion being separated under pressure and then Seitz-filtered.

Both the culture medium filtrate and the cell-sap were used for skin tested in dialyzed and nondialyzed form. Dialysis was performed against running tap-water for twenty-four hours. Freeze-dried preparations of the dialyzed and nondialyzed materials were prepared and were reconstituted in carbol-saline for quantitative comparative tests.

The uninoculated culture medium was employed for control tests in both dialyzed and nondialyzed form.

Serologic Tests of Aspergillus Extracts with Rabbit Antisera

A rabbit antiserum was prepared against *A. fumigatus* by injecting ground dried mycelial mat in an aluminium phosphate solution (10 mg. per ml.) emulsified with an equal volume of Bayol F®[3] and Arlacel®.[4] Two weekly injections of 10 mg. of the fungal mycelium were followed by 50 and 120 mg. at weekly intervals. Agar-gel tests by the Ouchterlony method (9*a*) were performed with the rabbit antiserum and concentrated solutions (10 mg. per ml.) of the dialyzed freeze-dried extracts of four- to six-week-old culture medium filtrates of *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus*. Tests were also performed with the rabbit *A. fumigatus* antiserum and carbol-saline extracts of Cladosporium, Penicillium, Alternaria, and Candida. The usual procedure was to place the antiserum in the central well and the Aspergillus and other extracts in the outlying wells.

Inhibition tests were also performed by adding the test extracts to the antiserum in the central well and observing the effect on the production of precipitation lines by the extracts placed in the peripheral wells. The failure of expected precipitation lines to appear indicated that the extract added to the antiserum contained antigenic components which combined with antibody in the antiserum. This combination prevents the diffusion of antibody into the agar and thus inhibits precipitation by antigens diffusing from the peripheral wells. This inhibition of the precipitation line of extracts of one species by the addition of extracts of another to the antiserum is evidence that common antigenic components are present.

Antigenic and Allergic Components of A. fumigatus

Paper electrophoresis of concentrated solutions (20 mg. in 0.1 ml.) of dialyzed freeze-dried culture medium filtrate of *A. fumigatus* was performed in barbiturate buffer, pH 8.6, for sixteen hours, with a current of 2 milliamperes per paper, the strips being 4 cm. in length. The paper strips were then developed with naphthalene black and scanned with a densitometer to show the spread of material through the paper.

Full-length strips of the paper after electrophoresis of the Aspergillus extract were also cut and laid flat upon the surface of plates of agar-gel, parallel to a trough of equal length in the agar into which rabbit antiserum against *A. fumigatus* was placed. The antigenic components diffused into the gel from the paper, and the production of precipitation lines was observed. Corresponding transverse sections of paper were cut and eluted. The eluted material was dialyzed, freeze-dried, and reconstituted in the original volume of the extract used for electrophoresis, for skin testing in man.

[3] Light liquid paraffin.

[4] Highly purified sorbitan esters of fatty acids.

Serologic Tests with Human Sera

A wide range of 60 specimens of serum from patients with and without *A. fumigatus in the* sputum, and with and without allergic hypersensitivity to Aspergillus extracts, were tested by the Ouchterlony agar-gel method (9*a*).

Dialyzed, freeze-dried extracts of culture medium filtrates and cell-sap of the various Aspergillus species listed above were reconstituted in concentrated solution (10 mg. per ml.) in carbol-saline and were employed for the testing. A number of serum specimens were also tested with carbol-saline extracts of other fungi, including *Cladosporium herbarum*, *Penicillium notatum*, *Alternaria sp.*, and *Candida albicans*.

RESULTS

Comparison of Skin Test Reactions to Various Aspergillus Species

Of 170 asthmatic patients, 117 gave positive reactions to one or more allergens. Of the 117 positive reactors, 41 gave positive reactions to the Aspergillus extracts. In almost all instances the Aspergillus-sensitive subjects reacted to one or more of the commoner allergens tested.

Carbol-saline (mycelial) extracts: These are conventional skin test extracts and may show considerable variation in potency in a series of extracts of any one particular material. The results of prick tests with these extracts are given in table 3 and show that Aspergillus-sensitive subjects tend to react to more than one member of the Aspergillus genus.

Culture medium filtrate preparations: Control tests with uninoculated medium, both dialyzed and nondialyzed, gave reactions of 2 and 3 mm. in diameter, respectively, in 2 of 34 asthmatic patients. In 20 of these patients positive reactions were elicited by the culture medium filtrates, both dialyzed and nondialyzed, of one-month-old cultures. The results in table 4 show that most of the sensitive subjects reacted to all of the species tested and that *A. fumigatus* and *A. nidulans* provided the most potent extracts.

Cell-sap preparations: Twelve of 29 asthmatic patients gave positive reactions to almost all of the extracts tested in almost all cases, as shown in table 5. *A. fumigatus* gave the most potent extract, followed by that from *A. nidulans*.

Comparison of cell-sap and culture medium filtrate: A quantitative comparison was made of cell-sap and culture filtrate preparations which were reconstituted from freeze-dried material into comparable concentrations in carbol-saline.

The results of tests in 10 sensitive subjects are shown in table 6. The culture filtrate was more potent than the cell-sap, and in each case the dialyzed material was more potent than the non-dialyzed.

The Aspergillus-sensitive subjects thus reacted to most of the 6 species tested, *A. fumigatus* giving the most frequent and vigorous reactions, followed by *A. nidulans*. Culture medium filtrates are a good and readily available source of non-dialyzable allergen. All of the patients with pulmonary eosinophilia reacted on skin testing with *A. fumigatus*. Two other asthmatic subjects reacted only to the *A. nidulans* extract.

Nature of skin test reactions: In 6 subjects tested with the more potent culture filtrate and cell-sap preparations, the immediate weal reactions were followed after an interval of three to twelve hours by "late" reactions, consisting of local swelling, which sometimes extended over a large part of the forearm, with poorly defined but definite central induration. This reaction came on hours after the weal had disappeared completely. The swelling was maximal at twenty-four hours and then began to resolve. It differed from the typical tuberculin reaction which lacks the immediate weal component, becomes evident at twenty-four hours, and is maximal at forty-eight to seventy-two hours. The tuberculin reaction also shows more clearly defined and tense induration and greater erythema.

In one subject in whom concurrent "late" reactions to Aspergillus extract and tuberculin reactions of comparable size were elicited, the difference between the two types of reaction was confirmed histologically. At six hours the Aspergillus reaction showed more marked edema and cellular infiltration than the tuberculin reaction. The cellular infiltration of the Aspergillus reaction was almost entirely composed of eosinophils, whereas the tuberculin reaction showed the

TABLE 3

SKIN TEST REACTIONS
ASPERGILLUS MYCELIAL EXTRACTS

Number of Species Tested	Number of Patients Tested	Positive Prick-test Reactions to the Following Number of Species Extracts:					
		1	2	3	4	5	6
2	3		3				
3	4		2	2			
4	22	5	4	8	5		
5	11		4	1	5	1	
6	4				1	2	1

Frequency of positive reactions to each extract in subjects tested: *A. fumigatus:* (34/40); *A. flavus:* (18/19); *A. glaucus:* (4/14); *A. niger:* (24/41); *A. nidulans:* (22/31); *A. terreus:* (22/34).

TABLE 4

SKIN TEST REACTIONS
ASPERGILLUS CULTURE FILTRATE (1 MONTH)

	Prick-test Reactions in 20 Subjects		
	Number Positive	Average diameter of weals (mm.) for entire group	
		Filtrate	Dialyzed Filtrate
A. fumigatus	18	4.7	4.8
A. flavus	18	2.7	2.6
A. nidulans	17	5.8	5.4
A. niger	16	2.3	2.2
A. terreus	16	2.4	2.9

TABLE 5

SKIN TEST REACTIONS
ASPERGILLUS CELL SAP
(1 Month Mycelium)

Prick-test Reactions in 12 Subjects

	Number Positive	Average Diameter Weals (mm.) for Entire Group
A. fumigatus	12	6.8
A. flavus	11	2.8
A. nidulans	10	5.4
A. niger	10	2.1
A. terreus	10	2.3

TABLE 6

SKIN TEST REACTIONS
COMPARISON OF A. FUMIGATUS CELL SAP AND CULTURE FILTRATE
(1 Month Freeze-dried)

Positive Prick-test Reactions in 10 Subjects

	Average Diameter Weal (mm.)	
	10 mg./ml.	1 mg./ml.
Cell sap dialyzed	3	7
Cell sap nondialyzed	2.2	4.2
Culture filtrate dialyzed	5.7	8.8
Culture filtrate nondialyzed	2.7	5.5

incipient typical round-cell infiltration. The Aspergillus reaction at forty-eight hours showed edema and intense eosinophilic infiltration in contrast to the perivascular round-cell aggregates of tuberculin reactions. Biopsy sections of late Aspergillus reactions taken at twenty-four hours in 2 other subjects showed changes similar to those described above.

In 2 subjects, giving "late" reactions, the tests were repeated while they were under treatment with corticosteroids. A vigorous immediate wealing reaction resulted, but there was a complete absence of the "late" reaction. Further testing after the cessation of the corticosteroid treatment elicited "late" reactions once again.

The nature of the "late" Aspergillus reaction suggests that it may be an Arthus type of reaction, which in experimental animals is related to the presence of precipitating antibody in the serum. The features distinguishing these reactions from typical delayed tuberculin reaction were the speed of development and resolution and the macroscopic and microscopic appearances.

Serologic Tests with Rabbit A. Fumigatus Antiserum

In the agar-gel tests *A. fumigatus* extracts produced two main precipitation lines indicating the presence of at least 2 antigens. Extracts of *A. flavus*, *A. nidulans*, and *A. niger* all produced one precipitation line, while *A. terreus* did not produce any precipitation lines. The single precipitation line produced by extracts of *A. flavus*, *A. nidulans*, and *A. niger*, when these were placed in adjoining wells, met and fused without crossing; that is, they gave a "reaction of identity." The single line of these species likewise gave a "reaction of identity" with one of the precipitation lines of *A. fumigatus*, thereby indicating that there is at least one common antigen in the Aspergillus species extracts which were tested.

In tests with the *A. fumigatus* antiserum and carbol-saline extracts of the other fungi, a single common precipitation line, common also to one of the Aspergillus extract lines, was produced by Cladosporium and Penicillium. No lines were produced by the Alternaria and Candida extracts. This shows that Cladosporium and Penicillium extracts contain a common antigen which is also present in the Aspergillus extracts.

Agar-gel inhibition tests: The addition of *A. fumigatus* extract to the antiserum in the central well inhibited the formation of the precipitation lines which it was capable of producing and also the single common precipitation line produced by the other Aspergillus species. When these other extracts were added to the antiserum, each of them inhibited the formation of the single common precipitation line of the others. The *A. terreus* extract, while unable to produce a precipitation line, nevertheless also inhibited the single common precipitation line of the other species when it was added to the antiserum.

It should be noted that all of the various species were tested against an antiserum produced in response to *A. fumigatus*. The precipitation line which they produced and the inhibition tests indicate that there is at least one main common antigen present in them and in *A. fumigatus*. Furthermore, *A. fumigatus* has additional antigens not present in the others.

Inhibition tests were also performed by adding Aspergillus, Cladosporium and Penicillium extracts, respectively, to the *A. fumigatus* antiserum and observing the effect on the single precipitation line common to all three. In each case the common precipitation line of the extract added to the antiserum and of the other two extracts was inhibited. The Cladosporium and Penicillium extracts inhibited only the common line produced by the Aspergillus extract, leaving the other Aspergillus precipitation lines intact; these results indicate the presence of common antigenic components in the three fungi.

Electrophoretic Investigation of A. Fumigatus Extract

Scanning of the stained paper strip after electrophoresis of the *A. fumigatus* culture medium filtrate showed a separation into two main peaks, as illustrated in figure 1*A*.

The separation of at least two antigenic components by the electrophoresis was also shown by the agar-gel test. The strip of paper through which the extract had been passed by electrophoresis contained at least two antigenic components. This was shown by the appearance of two precipitation lines in the zone of agar-gel which separated the electrophoresis paper lying on the surface of the agar and the equally long trough containing rabbit *A. fumigatus* antiserum, which ran parallel to the paper strip (figure 1*B*).

Since the scanning pattern and the agar-gel

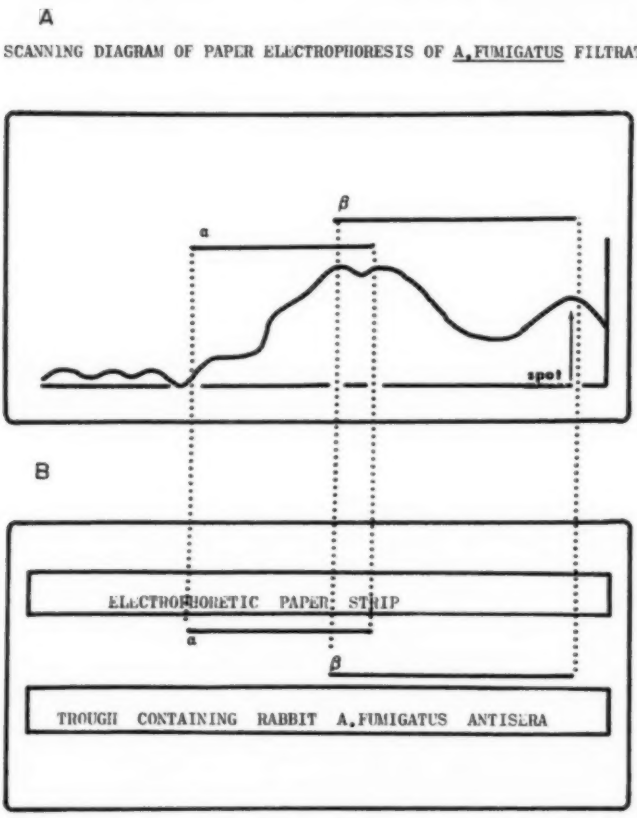

FIG. 1. Precipitation reactions with rabbit *A. fumigatus* antiserum, produced by antigenic components diffusing through the surface of an agar-gel plate from the full-length electrophoretic paper strip. The position of the precipitation lines in the agar-gel is projected onto the scanning diagram to show the relationship of the precipitating antigens to the spread of the filtrate by the electrophoresis.

tests were derived from the full length of the paper strip in each case, it was possible to appose the two sets of findings and to relate the position of the antigenic components as shown by the agar-gel precipitation to the peaks on the scanning pattern, as shown in figure 1*A* and *B*. It appears that among the antigenic components of the filtrate there are at least two protein-containing antigens and that the two peaks represent the separation of these two antigens by the electrophoresis. The association of carbohydrate with these protein-containing antigens was demonstrated by staining precipitation lines in agar gel by the periodic acid-Schiff method.

The next step was to cut three transverse sections of the electrophoresis paper corresponding to the position of the antigens, called for convenience the α and β components, and to the strip of paper which gave no reaction. These strips were eluted with water and the eluates were dialyzed and then freeze-dried. This material was reconstituted in carbol-saline, and prick tests were performed in man. The eluates of both of the strips of paper containing the α and β components excited positive prick-test reactions, whereas the third strip gave negative or minimal reactions. These findings indicate that among those components of the extract which are antigenic for rabbits there are components which are allergenic for man.

Serologic Test with Human Sera

The results of the agar-gel diffusion tests with human sera and Aspergillus extracts are given in table 7. The presence of precipitating antibody against *A. fumigatus* extracts was shown by the appearance of two or more lines in the serum of 5 patients, and 4 specimens of the serum gave precipitation lines with *A. nidulans* or *A. flavus* as well. A single precipitation line was given by the serum of 8 others.

TABLE 7

SEROLOGIC TESTS WITH HUMAN SERA AND ASPERGILLUS ANTIGEN

Number of Patients	Sputum *A. fumigatus* Cultured	Immediate Skin Test Reaction	"Late" Skin Test Reaction	Bronchial Test Reaction Aspergillus Extract	Precipitins
13	+	10 +ve 2 −ve (1NA)*	3	7 +ve 2 −ve (4NA)	+
14	+	+	3	10 +ve 1 −ve (3NA)	0
8	+	0	0	0	0
6	0	+	0	2 +ve 2 −ve (2NA)	0
5	NA	+	0	2 −ve (2NA)	0
13	NA	8 −ve (5NA)	0	NA	0

* NA = not available or test not performed.

All of the 13 patients with precipitating antibody in their serum had *A. fumigatus* cultured from their sputum. Positive skin test reactions in 10 patients and positive bronchial tests in 7 of these were elicited by Aspergillus extracts. There were, however, 2 patients with precipitating antibodies in their serum and with *A. fumigatus* in their sputum who did not react to Aspergillus extract on skin and bronchial testing. That is, the precipitating antibody was present in the absence of evidence of allergic hypersensitivity to Aspergillus extracts. One of these patients had a mycetoma and the other was asthmatic. In a further recent asthmatic patient, not included in table 7, precipitating antibody has been found in the absence of allergic hypersensitivity and, as yet, *A. fumigatus* has not been cultured from the sputum.

No precipitins were found in the serum of 14 other patients who also had *A. fumigatus* in the

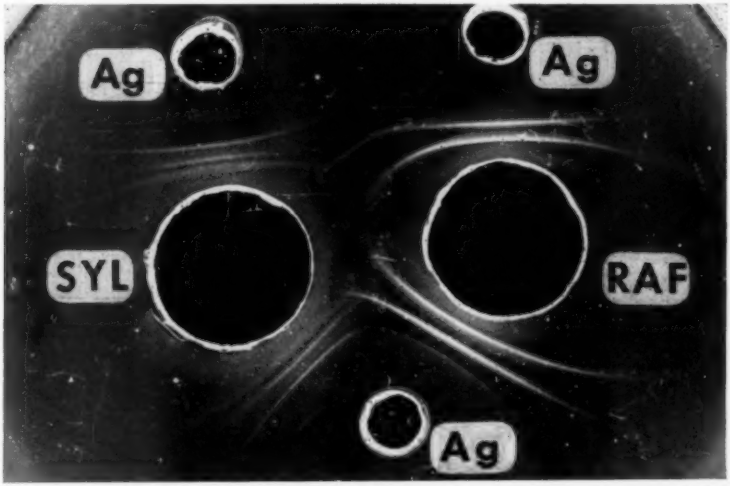

FIG. 2. Comparison of rabbit *A. fumigatus* antiserum and human serum in agar-gel diffusion test with *A. fumigatus* cell sap.

Ag = Wells containing *A. fumigatus* cell sap, dialyzed, freeze-dried, and reconstituted in a concentration of 10 mg. per ml.

SYL = Serum of a patient with bronchopulmonary aspergillosis.

RAF = Rabbit *A. fumigatus* antiserum.

The *A. fumigatus* preparation used on this occasion produced three precipitation lines, with both sera (top of plate). The outermost lines in relation to the antibody wells for both sera meet and fuse without crossing, i.e., give a "reaction of identity" (bottom of plate).

sputum and were hypersensitive to Aspergillus extracts. No precipitins were found in the serum of the remaining patients tested, some having *A. fumigatus* in the sputum but no evidence of hypersensitivity, and vice versa. Yet others, selected at random, with negative skin tests, whose sputum had not been cultured, also had no precipitins.

The serum of one patient also gave a single common precipitation line with the extracts of Cladosporium and Penicillium, but not of Alternaria and Candida. This single line was common also to one of the Aspergillus lines. Inhibition tests were performed with this patient's serum and with extracts of Aspergillus, Cladosporium, and Penicillium. In each case the common precipitation line of the extract added to the patient's serum and of the other two extracts was inhibited. As with the tests with the rabbit *A. fumigatus* antiserum, these results indicate the presence of common antigenic components in the three fungi. None of the remaining sera containing precipitins to *Aspergillus* listed in table 7 gave precipitation lines with any of the other fungal extracts.

Comparison of human and rabbit A. fumigatus antiserum: A comparison of a human serum containing precipitating antibodies to *A. fumigatus* and a rabbit *A. fumigatus* antiserum was made by agar-gel tests, with the results shown in figure 2. In this instance the *A. fumigatus* cell-sap excited the production of at least four precipitation lines with the human and four with the rabbit antiserum. Of these lines there was fusion without crossing, that is, "reaction of identity" between one of the lines produced with the rabbit and human sera, respectively, thus indicating that one of the antigens in the cell-sap had been antigenic and had induced the production of a specific precipitating antibody in both man and the rabbit.

Discussion

The possibility that hypersensitivity to *A. fumigatus* plays an important part in the production of pathogenic manifestations is supported by clinical and immunologic evidence.

Comparison of the prevalence of *A. fumigatus* in the sputum shows that it occurs with greater frequency in asthmatic than in nonasthmatic patients. Enhanced absorption of pollen grains through the nasal mucosa of sensitized as compared with nonsensitized guinea pigs has been reported by Strömme (9). This suggests a possible explanation for the greater frequency with which *A. fumigatus* is found in the sputum of the typically allergic asthmatic, namely, that the allergic reaction to the inhaled spores may lead to their retention in the respiratory tract. Preexisting lung damage also permits colonization by *A. fumigatus*, as in the cases of mycetomata. An inquiry into the occupations of the patients did not reveal any definite exposure to sources generally regarded as important, for example, mouldy hay. The ubiquity of the fungal spores and the widespread extent of allergic hypersensitivity to them must make their encounter and reactions to them a common event.

The respiratory manifestations associated with *A. fumigatus* in the sputum of the patients described in this investigation may be considered as follows: with allergic hypersensitivity, and without allergic hypersensitivity.

Allergic hypersensitivity may produce respiratory disturbances in two ways. First, there may be reactions to exposure to fungal antigens, with the production of asthma, rhinitis, and conjunctivitis, in the same way that persons sensitive to other allergens, e. g., pollen, react on exposure to them. Second, the hypersensitivity appears to be related to the appearance of transient infiltrations in the lungs with blood eosinophilia. The features of this condition have been described by Hinson and others (5). It seems likely that *A. fumigatus* may produce bronchial damage in these cases by plugging the bronchi and by the severe hypersensitivity reaction which may be present. The manifestations of pulmonary eosinophilia occurred in the present series with greater frequency in long-standing asthmatics, most of whom showed widespread allergic hypersensitivity to other common allergens. The reactions excited by direct bronchial tests with Aspergillus extracts in the patients with pulmonary eosinophilia confirm the presence of hypersensitivity of the bronchial tract itself to the Aspergillus extracts.

Among the patients without allergic hypersensitivity were patients with pulmonary mycetomata and even others in whom the fungus appeared to be saprophytic and perhaps unimportant.

The nature of the "late" skin test reactions to the Aspergillus extract suggests that they are

of the Arthus type. These late reactions, which were sometimes extensive in response to a single superficial prick test, showed edema and an intense eosinophilic infiltration, resembling the histologic features of the few cases of Löffler's syndrome reported in the literature (10).

Arthus reactions in experimental animals are closely correlated to the titer of precipitating antibody in the serum (11–13). While, in general, precipitating antibody has been repeatedly shown to be necessary for the active or passive production of Arthus reactions, there have been reports which question the close correlation of Arthus reactivity and precipitin titer (14, 15). Using collodion-particle techniques, Cannon and Marshall (12) claimed to demonstrate precipitins more frequently during periods of Arthus reactivity. It has also been found that injections of the antigen into sensitized rabbits depressed the precipitin titer and, with it, the Arthus reactivity, and in young guinea pigs high titers of precipitating antibody could be associated with low degrees of Arthus reactivity (12).

These experimental observations may have some bearing on the late "Arthus" reactions in the patients of the present study and their relationship to the demonstration of precipitating antibody. "Late" reactions were observed in 3 of the 12 patients with precipitins who were skin tested. It should be stressed that the prick test was of a limited nature. Indeed, in one patient the "late" reaction was elicited by subcutaneous injection of Aspergillus extract for treatment purposes. The degree of hypersensitivity present and the possibility of undesirable bronchial or systemic reactions make it undesirable to try to produce "late" reactions by deliberate injection. Of the 3 patients giving "late" reactions in whose serum precipitins were not found, 2 had received injections of Aspergillus extract in recent months. This may have accounted for the absence of precipitins, although it is also possible that improved antigens will demonstrate precipitins more frequently.

The role of the precipitating antibody in patients with *A. fumigatus* in the sputum appears to be related to the manifestations of pulmonary eosinophilia. The pulmonary infiltrations could be compared with the "late" Arthus type of reactions to skin testing. On the one hand, precipitins could be present in the absence of allergic hypersensitivity to Aspergillus extracts; and, on the other hand, in 2 asthmatic patients, although treatment with Aspergillus extracts was followed by the disappearance of precipitins from the serum, bronchial hypersensitivity to inhalations of Aspergillus extract was unaltered. These findings suggest that at least two types of antibody against Aspergillus extracts may be concerned in these patients. First, nonprecipitating skin-sensitizing antibody of the type found, for example, in pollen hypersensitivity and termed reagin, mediating the immediate skin and bronchial test reactions and responsible for the rhinitis and asthma; second, the precipitating antibody mediating the "late" Arthus skin reactions and the pulmonary eosinophilia.

It is of interest that Stanley (16) has reported that a lipoid extract of *A. fumigatus* augmented the antibody response to other antigens, at the same time provoking a monocytosis. This stimulation of the reticuloendothelial system by the lipoid from *A. fumigatus* may perhaps contribute to its antigenic potency in man.

The antigenic relationship between Aspergillus Cladosporium, and Penicillium extracts shown by the precipitation reactions with rabbit *A. fumigatus* antiserum and with human serum raises many problems and is being investigated in more detail.

The profusion with which *A. fumigatus* was cultured from the sputum was not necessarily related to the clinical manifestations. In some of the hypersensitive patients, frequent examinations of sputum were required before the fungus could be found. The importance of supporting evidence of an immunologic nature in these patients makes it essential to investigate their hypersensitivity toward Aspergillus extracts and to examine their serum for precipitins. The evidence is accumulating which suggests that there is a close association between the presence of *A. fumigatus* in sputum, precipitins in serum, positive skin test reactions, and pulmonary infiltrations. With adequate skin test preparations, a prick test may be of value for diagnostic purposes in patients with pulmonary eosinophilia. A negative reaction makes it unlikely that Aspergillus infection is responsible. A positive reaction should, if possible, be confirmed by bronchial tests, since skin test hypersensitivity may be present without reactions to bronchial tests, with the preparations and concentrations of test material considered safe at present.

Summary

The significance of the culture of *A. fumigatus* from the sputum of 145 of a total of 2,080 patients has been investigated. *A. fumigatus* was found in the sputum of asthmatic subjects with a statistically significant, greater frequency than in the sputum of patients with other respiratory diseases.

Detailed investigation of 27 patients with *A. fumigatus* in the sputum showed two groups. The first group of 16 were hypersensitive to bronchial and skin tests with Aspergillus extracts and had episodes of pulmonary eosinophilia. The second group of 11 did not react to bronchial tests, gave infrequent reactions to skin tests, and did not have episodes of pulmonary eosinophilia.

Skin-prick tests showed that one-month-old culture filtrates provided the most potent material, followed by cell sap expressed from mycelial mats, and finally by conventional carbol-saline extracts of dried mycelium. Patients sensitive to Aspergillus extract on skin testing reacted in almost all cases to extracts of *A. fumigatus*, *A. flavus*, *A. glaucus*, *A. nidulans*, *A. niger*, and *A. terreus*, that is, to all of the species tested. *A. fumigatus* extract gave positive reactions in all patients with *A. fumigatus* in the sputum and with pulmonary eosinophilia. In addition to immediate weal reactions in sensitive subjects, "late" reactions considered to be of the Arthus type appeared in a number of subjects.

Precipitating antibodies to Aspergillus extracts were demonstrated in the serum of 13 patients, all of whom had *A. fumigatus* in the sputum. Skin and bronchial tests showed hypersensitivity to Aspergillus extracts in 10 of these patients. No precipitins were found in 14 other similar patients, nor in 32 patients with and without the fungus in their sputum, and with or without allergic hypersensitivity to Aspergillus extracts.

The *A. fumigatus* extracts contained at least two main antigens as shown by agar-gel diffusion tests with human sera and with rabbit *A. fumigatus* antisera. The other Aspergillus species extracts have at least one antigen in common with *A. fumigatus*, as shown by tests with rabbit antisera.

Precipitins against common antigenic components in Aspergillus, Cladosporium, and Penicillium were found in rabbit *A. fumigatus* antiserum and in a human serum.

The findings suggest that hypersensitivity to *A. fumigatus* is related to the appearance of pulmonary eosinophilia and that the presence of precipitating antibody may mediate the production of Arthus-like reactions in the lungs.

Sumario

El Significado Clínico e Inmunológico del Aspergillus Fumigatus en el Esputo

Se ha investigado la significación del cultivo del *A. fumigatus* del esputo de 145 enfermos entre un total de 2,080. El *A. fumigatus* fué encontrado en el esputo de los asmáticos con una frecuencia mayor, y estadísticamente importante, que en el esputo de los sujetos que tenían otras enfermedades del aparato respiratorio.

Una investigación detenida de 27 enfermos que tenían *A. fumigatus* en el esputo reveló dos grupos. El primer grupo de 16 era hipersensible a las pruebas bronquiales y cutáneas con extractos de aspergilo y mostraba episodios de eosinofilia pulmonar. El segundo grupo, de 11, no reaccionó a las pruebas bronquiales, acusó reacciones infrecuentes a las pruebas cutáneas y no tuvo episodios de eosinofilia pulmonar.

Pruebas de pinchazos cutáneos revelaron que los filtrados de un cultivo de un mes suministraban la substancia más potente, viniendo después el jugo celular exprimido de las capas de micelio, y a lo último los extractos carbol-salinos corrientes de micelio desecado. Los enfermos sensibles al extracto de aspergilo a la cutirreacción reaccionaron en casi todos los casos a extractos de *A. fumigatus*, *A. flavus*, *A. glaucus*, *A. nidulans*, *A. niger* y *A. terreus*, es decir, a todas las especies ensayadas. El extracto de *A. fumigatus* produjo reacciones positivas en todos los enfermos que tenían *A. fumigatus* en el esputo y eosinofilia pulmonar. Además de las reacciones inmediatas con ronchas en los sujetos sensibles, aparecieron en varios individuos reacciones "tardías," que se considerearon como de la forma Arthus.

Se descubrieron anticuerpos precipitantes para los extractos de aspergilos en los sueros de 13 enfermos, todos los cuales tenían *A. fumigatus* en el esputo. Las pruebas cutáneas y bronquiales revelaron hipersensibilidad a los extractos de aspergilos en 10 de estos enfermos. No se descubrieron precipitinas en otros enfermos semejantes ni tampoco en 32 sujetos con o sin el hongo en el esputo y con o sin hipersensibilidad alérgica a los extractos de aspergilos.

Los extractos de *A. fumigatus* contenían a lo menos dos antígenos principales, según demostraron las pruebas de difusión con agar-gel con sueros humanos y con antisueros de *A. fumigatus* de conejo. Los extractos de otras especies de aspergilos poseen a lo menos un antígeno en común

con el *A. fumigatus*, según revelaron las pruebas con antisueros de conejo.

En el antisuero de *A. fumigatus* de conejo y en un suero humano, se encontraron precipitinas contra los componentes antigénicos comunes de Aspergillus, Cladosporium y Penicillium.

Los hallazgos denotan que la hipersensibilidad al *A. fumigatus* se relaciona con la aparición de eosinofilia pulmonar y que la presencia de un anticuerpo precipitante puede intervenir en la producción de reacciones parecidas a la Arthus en los pulmones.

Resume

Signification clinique et immunologique de Aspergillus fumigatus dans l'expectoration

La signification de la culture de *A. fumigatus* provenant de l'expectoration de 145 malades sur un nombre total de 2.080 patients a été étudiée. *A. fumigatus* a été décelé dans les crachats des asthmatiques plus fréquemment que dans les crachats de malades atteints d'autres affections respiratoires.

Une étude détaillée de 27 patients, dont l'expectoration recélait *A. fumigatus* révélait l'existence de deux groupes. Le premier groupe de 16 réagissait aux tests bronchiques et cutanés aux extraits d'Aspergillus et présentait une éosinophilie épisodique. Le second groupe de 11 ne réagissait pas aux tests bronchiques, ne présentait que rarement des réactions aux tests cutanés et n'avait pas eu d'éosinophilie pulmonaire épisodique.

Par ordre d'efficacité, les tests cutanés par piqûre révélaient que les filtrats de cultures d'un mois constituaient le matériel le plus actif; venait ensuite la substance cellulaire exprimée d'aggrégats mycéliens et enfin les extraits carbo-salins classiques de mycélium sec. Les patients ayant une sensibilité cutanée à l'extrait d'Aspergillus réagissaient dans presque tous les cas aux extraits de *A. fumigatus*, *A. flavus*, *A. glaucus*, *A. nidulans*, *A. niger* et *A. terreus*, c'est-à-dire à toutes les espèces testées. L'extrait de *A. fumigatus* donnait des réactions positives chez tous les malades dont les crachats recélaient *A. fumigatus* et qui présentaient une éosinophilie pulmonaire. Outre les réactions papuleuses immédiates survenant chez les sujets sensibles, des réactions "tardives" considérées comme étant du type Arthus se manifestaient chez un certain nombre de malades.

La présence d'anticorps précipitant les extraits d'Aspergillus a été démontrée dans le sérum de 13 patients; l'expectoration de tous ces malades contenait le *A. fumigatus*. Les tests cutanés et bronchiques montraient une hypersensibilité aux extraits d'Aspergillus chez 10 de ces malades. Il n'a pas été décelé de précipitines chez 14 autres malades semblables, ni chez 32 patients dont les crachats recélaient ou non le fungus et qui présentaient ou non une hypersensibilité allergique aux extraits d'Aspergillus.

Les extraits de *A. fumigatus* contenaient au moins deux des antigènes, comme l'ont montré les tests de diffusion en gel agar effectués avec le sérum humain et l'antisérum *A. fumigatus* de lapin. Les extraits d'autres espèces d'Aspergillus ont au moins un antigène en commun avec *A. fumigatus*, comme le révèlent les tests effectués avec les antisérums de lapin.

Des précipitines de constituants antigéniques d'Aspergillus, Cladosporium et Pénicillium ont été découvertes dans l'antisérum *A. fumigatus* de lapin et dans un sérum humain.

Ces résultats suggèrent que l'hypersensibilité à *A. fumigatus* est en relation avec l'apparition de l'éosinophilie pulmonaire et que la présence d'un anticorps précipitant peut intervenir dans la production des réactions pulmonaires du type Arthus.

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CLINICAL AND PHYSIOLOGIC RESULTS FOLLOWING RADIOACTIVE IODINE (I^{131}) IN THE TREATMENT OF CHRONIC PULMONARY INSUFFICIENCY[1, 2]

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Introduction

The treatment of chronic pulmonary insufficiency is often discouraging despite the judicious use of all standard forms of therapy. In patients with pulmonary disability, dyspnea occurs when the breathing requirement approaches the breathing capacity. It is reasonable that reduction of metabolic demands of the body by inducing hypothyroidism with radioactive iodine (I^{131}) will decrease breathing or oxygen requirements and thereby lessen dyspnea. For the same reason, reduction in the carbon dioxide level might be expected along with other advantageous physiologic alterations. One of the writers (W. F. H., Jr.) and Dr. L. Q. Hair of Augusta, Georgia, in 1952 administered radioactive iodine to eight patients with severe chronic pulmonary insufficiency. The results were so favorable that the present study was begun. Subsequently other reports (1–3) of encouraging results have been published.

Material and Methods

Treatment of patients with severe chronic pulmonary insufficiency was begun in June, 1954, and during the following year 23 patients were selected for this study. All were badly disabled despite intensive and long-term therapy by competent physicians using conventional measures plus corticotropin or cortisone in several cases. Symptoms had been severe for at least two years in all patients, and the majority of this group had been unable to carry on gainful occupation for one to five years. A few were able to do light work for a few hours a day, and 2 managed sedentary jobs. All were showing evidence of progressive incapacitation. The probability of achieving a worthwhile result was admittedly small because their clinical and physiologic status was so poor. Many of the patients had such small pulmonary reserve that the slightest exertion, such as dressing, walking across the room, and even talking or eating, was enough to precipitate marked respiratory distress. Clinical observations and laboratory and physiologic determinations were made before treatment and at approximately two-month intervals thereafter for six to twenty-six months (average of sixteen months). In November, 1957, a questionaire regarding the patient's condition (two and one-half to three and one-half years after therapy) was sent to each referring physician.

Routine history, physical and laboratory examination, including protein-bound iodine, cholesterol, electrocardiogram, and chest roentgenogram were done prior to therapy. I^{131} uptake studies were obtained by administering a tracer dose and twenty-four hours later measuring the per cent uptake with a Geiger-Mueller counter. Pulmonary function was evaluated by the following measurements performed in accordance with standard procedures and compared with the predicted normal (4): lung volumes and maximal breathing capacity; timed vital capacity (5); alveolar nitrogen after breathing oxygen for seven minutes (index of intrapulmonary mixing); ventilation and gas exchange. The latter were performed with the patient in the supine position and breathing room air. Arterial blood was obtained anaerobically from an indwelling needle in the brachial artery simultaneously with collection of expired air. The arterial oxygen and carbon dioxide tensions were measured in duplicate by the Riley bubble equilibration technique (6) with 4 mm. checks required. Expired air was analyzed with the Scholander apparatus (7) with duplicate determinations required to check within 0.03 per cent. Arterial oxygen and carbon dioxide contents and oxygen capacities were determined manometrically by the method of Van Slyke and Neill (8) with checks to 0.2 per cent. The mean alveolar oxygen tension was calculated according to the alveolar equation (9), and the alveolar-arterial oxygen difference was determined. Physiologic dead space was determined, using the Bohr equation (10), and the alveolar ventilation was calculated using the formula (tidal volume − dead space) times respiratory rate.

After the initial studies were completed, approximately 25 mc. of I^{131} or a placebo were administered orally, and the patient was instructed to return for follow-up examination in two months,

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further treatment and management depending on response to therapy. Neither the internist nor the physician in charge of pulmonary function studies was aware which persons received placebos. All patients were placed on prophylactic doses of 0.5 gm. of sulfisoxazole (Gantrisin®) twice daily and, although the majority were receiving bronchodilators of various types, no additional medication or change in treatment was prescribed.

One of the writers (B.S.G.) made all clinical evaluations of improvement, which was graded excellent (4+), good (3+), fair (2+), poor (1+), or none (0). Factors such as decrease in dyspnea, increase in exercise tolerance, and decrease in severity and number of asthmatic attacks entered into the evaluation of the degree of improvement.

Results

All 23 patients, ranging in age from seventeen to seventy-seven years (average age of fifty years), were diagnosed as having chronic obstructive emphysema as evidenced by physical and roentgenographic examination and pulmonary function studies. In addition, 2 patients had far advanced silicosis and 3 had pulmonary tuberculosis, one with active and two with arrested disease; 4 patients had had typical allergic bronchial asthma since childhood, whereas so-called "asthmatic attacks" played a prominent role in the disease of 5 patients. Three had extensive bilateral bronchiectases proved by bronchogram.

The average total quantity of I^{131} per patient was 40.1 mc. with a range of 17.5 to 75.0 mc. An average of 1.9 doses was administered per patient with a range of 1 to 4 doses. Although hypometabolism was never considered a serious problem in any case, 11 patients did require thyroid therapy. The onset of hypothyroidism occurred from six weeks to six months following the initial therapeutic dose and was usually accompanied by slight fullness or puffiness of the face. As myxedema progressed, complaints—although quite variable in type and degree—included lethargy, weakness, weight gain, drying of skin, dermatitis, paresthesias, constipation, aching of joints, hoarseness with lowering of the voice, and irritability or depression. Discomforts were usually easily controlled with 15 mg. or less of desiccated thyroid daily. Thyroid medication often caused an accentuation of the dyspnea. Patients themselves learned to regulate the dosage so that they obtained relief from the majority of the hypothyroid symptoms without increasing metabolism sufficiently to alter the beneficial effects of the I^{131}.

TABLE 1

Average Results of Pulmonary Function Tests in 23 Patients before Treatment with I^{131} and Average of Maximal Response after Treatment

	Before I^{131}	After I^{131}
Vital capacity (L.)	2.1 (56%)	2.3 (62%)
3-Second vital capacity	62%	63%
Maximal breathing capacity (L./min.)	26.7 (27%)	30.7 (32%)
Residual volume/total lung capacity (%)	71	64
Alveolar N_2 after 7-minute O_2	8.0%	7.3%
O_2 uptake (cc. per min. per M^2)	150	101
Ventilation (L. per min. per M^2)	5.8	4.0
Alveolar ventilation (L. per min. per M^2)	3.4	2.4
Alveolar O_2 tension (mm. Hg.)	93	108
Arterial O_2 tension (mm. Hg.)	73	86
Arterial CO_2 tension (mm. Hg.)	44	35

The clinical response was considered excellent in 7 patients (30 per cent), good in 2 (9 per cent), fair in 6 (26 per cent), and poor or none in 8 (35 per cent). Two patients died during the course of the study, one as a result of acute pulmonary insufficiency and the other from cerebral thrombosis following transurethral resection of carcinoma of the bladder. Subsequently, according to the report from local physicians, 4 more died, 3 directly as a result of their cardiopulmonary disease and one from an acute myocardial infarction. Thus, one-fourth of the entire group had died two and one-half to three and one-half years following the beginning of treatment.

The mean average of results in all 23 patients before treatment and the mean average of the maximal response after treatment are summarized in table 1. Values of lung volumes, timed vital capacity, maximal breathing capacity, and alveolar nitrogen after seven minutes of oxygen indicate the severity of the disease. In no case was the ventilatory capacity significantly altered following therapy. Some of the clinically improved patients could sustain expiratory effort

longer after treatment than before, thus increasing their respiratory reserve volume. The timed vital capacity and the maximal breathing capacity were not improved, and the increase in vital capacity and in expiratory reserve volume was regarded as an index of greater stamina rather than of improved ventilatory capacity. The intrapulmonary mixing index was above normal (more than 2.5 per cent N_2) at all times except in the 2 patients in whom silicosis was the primary disease and no improvement followed treatment.

In general, both the total and alveolar ventilation were above the predicted normal before treatment. Following therapy, in most cases a decrease occurred in both values but to a greater degree in total ventilation. The amount of oxygen removed per liter ventilated was in every case below normal and as a rule remained essentially unchanged. The physiologic dead space in almost every instance was above the normal limit of 30 per cent of the tidal volume, and a tendency for this to decrease after treatment was observed. A definite increase in mean alveolar oxygen tension, which was below normal before treatment, was seen to occur. The arterial oxygen tension was less than 92 mm. of mercury in all except 2 patients, and in the majority increased after treatment. The arterial pressure of carbon dioxide, above 44 mm. of mercury in 10 of the 23 patients before treatment, usually decreased as a result of treatment. The post-treatment levels of arterial pressure of carbon dioxide were as a rule maintained better than those of arterial oxygen tension. The alveolar-arterial oxygen difference was usually above normal before treatment and often increased after therapy.

Before treatment, oxygen uptake was near or slightly above the normal basal figure (120 to 145 cc. per min. per M^2) and in the majority of patients was reduced after treatment. In general, cholesterol, protein-bound iodine, and I^{131} uptake correlated well with oxygen uptake as indications of thyroid activity.

The response following treatment is illustrated in figure 1 in all patients in significant values, plotted according to the clinical evaluation of the patient at the time when the result was obtained. It is noted that the over-all response was similar regardless of whether or not clinical improvement occurred.

Certain patients warrant special mention.

The patient of *Case 5* was first admitted to this hospital four years prior to this study. At this time he was severely dyspneic, and catheter drainage of a large emphysematous bleb in the left upper lobe was performed with good clinical response. Disability continued, however, because of generalized bilateral emphysema. Difficulty gradually progressed and at the time I^{131} was administered he was almost completely incapacitated. Response from both the clinical and laboratory standpoint was good. It is interesting that, despite a known history of pulmonary disease and the presence of definite clubbing of fingers and toes, both the hematocrit and the electrocardiogram were normal.

The patient of *Case 9* was the only one with active tuberculosis in the series. He had been unable to work for the past two years because of dyspnea, and during this period had been hospitalized several times because of active pulmonary tuberculosis, each time leaving the hospital against medical advice. Because of his desperate clinical condition he was given I^{131} despite his history of uncooperativeness. Also at this time his private physician prescribed isoniazid-PAS. Improvement was truly amazing and the patient felt so well that, in spite of repeated warnings, he began working approximately ten to fourteen hours per day as a produce trucker. Fourteen months later he was admitted as an emergency to the state sanatorium where his clinical course was rapidly downhill, death occurring approximately ten months later.

It is possible that reduced metabolism played a role in the extension of the tuberculosis (11) although no adverse effects were observed in the 2 patients with arrested tuberculosis. At any rate, patients who are likely to be uncooperative and not heed medical advice should probably not be considered for this treatment. It is believed that hypoxic patients should also be warned against physical exertion even though they may not experience dyspnea (12–14).

Clinical response in the 2 patients with silicosis was good and, in addition, a definite and sustained increase in arterial oxygen tension occurred. Results were also favorable in 3 of the 4 patients with long-standing allergic bronchial asthma.

Although all patients presented interesting clinical courses, space will not permit each to be discussed separately. Three cases are presented, 2 in which treatment was considered successful and one in which it was ineffective.

Case Reports

Case 1. The patient, a 48-year-old white male, was first seen in May, 1954, with a history of

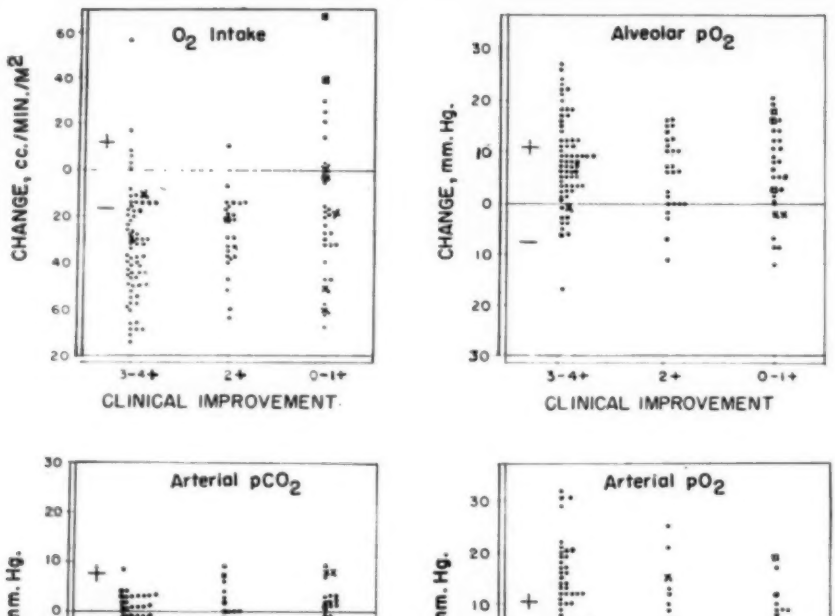

FIG. 1. Correlation of physiologic response with clinical improvement in 23 patients treated with I^{131}. Symbols represent results obtained after treatment as compared with pretreatment control values.

gradual onset of dyspnea starting approximately twelve years previously. For one year preceding his first admission he had been unable to work and he could not walk more than 100 feet without stopping to rest. Bronchial asthma had begun at ten years of age and bronchiectasis had been confirmed by bronchogram. The patient had quit smoking approximately one year ago. For a number of years he had been under the care of a chest specialist but, in spite of all routine measures, his condition progressed. Physical examination, roentgenogram, electrocardiogram, and pulmonary function studies were all consistent with the diagnosis of chronic obstructive pulmonary emphysema. He received 26.6 mc. of I^{131}.

Two months later the patient stated that approximately six weeks following treatment he noticed a decrease in dyspnea and an increase in exercise tolerance. There was progressive and marked improvement in exercise tolerance within a few months, and he claimed he could easily walk two and one-half miles without dyspnea. He gained 26 pounds, and, although hypothyroid by laboratory tests, he denied any symptoms. Six months following therapy he was employed as a look-out for the state forestry department which required his climbing an 80-foot tower at least twice daily. Also he had acquired a 27-year-old bride. In January, 1955, seven months after treatment, clinical myxedema became apparent, and he was given 15 to 30 mg. of dessicated thyroid

extract daily to counteract lethargy, periorbital puffiness, and paresthesias of hands and feet. Subsequent visits revealed that his improvement was maintained until July, 1956, approximately two years following treatment, when "weakness" had forced him to resign his job. He emphatically denied dyspnea. When thyroid dosage was increased, shortness of breath promptly returned, and he much preferred to tolerate the symptoms of the hypothyroid state. He was again admitted to the hospital in October, 1957, because of dermatitis over anterior neck, shoulders, arms, and hands, a biopsy of which showed changes typical of myxedema. Improvement of the dermatitis occurred when the dose of thyroid was increased. The patient's private physician reported in November, 1957, approximately three and one-half years following treatment, that the patient's condition remains the same, the marked improvement in dyspnea being maintained.

This patient's improvement was dramatic, and he is well content to exchange the myxedematous symptoms for severe dyspnea and disability. Unfortunately a balance could not be reached whereby both could be alleviated.

Case 2. The patient, a 45-year-old white male, was referred to the writers in May, 1954, because of severe respiratory distress, first noted about ten years previously. "Asthmatic attacks" began at approximately the same time. For the previous two years the patient had been forced to stay in bed almost completely. He had worked in a cotton mill for twelve years prior to the onset of his present illness, then in a butcher shop for five years when, because of dyspnea, he was forced to drive a delivery truck a few hours a day until disability became so marked he had to give this up. He had been treated with the usual standard measures without results. This man was extremely weak and cachectic and obviously dyspneic at rest. All clinical and laboratory data pointed to chronic obstructive pulmonary emphysema, and his electrocardiogram was consistent with cor pulmonale. In spite of an I^{131} uptake of only 8 per cent, which was thought possibly due to iodides in a cough preparation he had recently taken, he was given 26.6 mc. of I^{131}.

On subsequent visits the patient claimed he had received slight relief, but the improvement was thought to be doubtful. Ten months after treatment the I^{131} uptake was 18 per cent, and it was thought possible that the first dose had not been taken up by the thyroid because it was already saturated with iodine. He was therefore given another 26.6 mc. Two days later his local physician reported that he died suddenly as a result of acute pulmonary insufficiency and cardiac failure.

In retrospect it is believed that this patient had so little thyroid function before treatment that relief of symptoms could not be expected. Also his general physical condition was extremely poor. This probably represents one type of case in which I^{131} medication should not be attempted.

Case 17. A 17-year-old school boy was referred to the writers in January, 1955, for consideration of I^{131} therapy. He had had severe bronchial asthma since infancy and since October, 1954, had been so disabled that he was for the most part confined to bed. For the last two months he had been hospitalized and maintained on corticotropin. Physical examination revealed a mentally alert, but thin and physically underdeveloped boy, who was wheezing and obviously dyspneic at rest. In addition to the usual physical and laboratory findings and pulmonary function results of obstructive pulmonary emphysema, a "pigeon" chest deformity was present. An electrocardiogram indicated cor pulmonale. Eighteen millicuries of radioactive iodine were administered. When the patient returned two months later there had been marked improvement. Asthmatic attacks had decreased in frequency and severity, and corticotropin had been discontinued several weeks previously. He said that he had even been able to play a little baseball. Improvement continued, but in September, 1955, he had the worst asthmatic episode since treatment. At this time he was given 10 mc. of I^{131} in a divided dose at two-week intervals. Although asthma recurred on several occasions during the next year, he was able to attend school regularly and graduated from high school. In August, 1956, another 15 mc. of I^{131} were given prior to his entering the state university. In November, 1957, the patient's physician reported that he was in his second year of college and that his dramatic clinical improvement had continued. He was receiving only symptomatic treatment with bronchodilators and expectorants.

It is not at all clear why this excellent result was achieved. Although I^{131} uptake decreased to the hypothyroid range, there was little reflection of this in other laboratory data. Perhaps the allergic response was in some way altered.

Discussion

The enigma of these results is that, although the same physiologic response occurred in the majority of the patients, some were benefited whereas others were not. Conversely, a few improved clinically in spite of no favorable alteration in laboratory data.

It is believed that psychologic support was minimal. Patients were rarely seen more often than once in two months, and the experimental

nature of the treatment and the uncertainty of results were stressed. The time consumed and the care with which the various tests and examinations were performed naturally led the patients to believe that their physicians were taking a special interest in them. Also, the magic word "radioactive" is not to be underestimated. It would seem, however, that the psychologic element would decrease with the passage of time; but improvement, if it occurred, was usually sustained.

These results are gratifying when the severity and duration of disease in these patients are considered. Even a "fair" response meant a great deal to the patient. While it is difficult to predict which patient will respond favorably to therapy with I^{131}, it seems that the euthyroid patient with a decreased arterial oxygen tension and increased arterial pressure of carbon dioxide is most likely to be helped.

Summary

Twenty-three patients with severe pulmonary insufficiency were followed for two and one-half to three and one-half years after treatment with radioactive iodine. Clinical and physiologic results are presented. Clinical improvement was excellent in 7 patients (30 per cent), good in 2 (9 per cent), fair in 6 (26 per cent), and poor in 8 (35 per cent). Pulmonary function studies revealed no significant improvement in lung volumes or in the mechanics of breathing. In the majority of patients, oxygen uptake and I^{131} uptake were reduced, total and alveolar ventilation decreased, alveolar and arterial oxygen tension increased, and the arterial carbon dioxide tension decreased. The clinical improvement did not always show a correlation with the values determined in the laboratory.

Sumario

Resultados Clínicos y Fisiológicos Consecutivos al Yodo Radioactivo (I^{131}) en el Tratamiento de la Insuficiencia Pulmonar Crónica

A 23 enfermos de insuficiencia pulmonar grave se les mantuvo en observación de dos y medio a tres y medio años después del tratamiento con yodo radioactivo. Se presentan los resultados clínicos y fisiológicos. La mejoría clínica fué excelente en 7 enfermos (30 por ciento), buena en 2 (9 por ciento), mediana en 6 (26 por ciento) y mala en 8 (35 por ciento). Los estudios de la función pulmonar no revelaron mayor mejoría en los volúmenes pulmonares o la mecánica de la respiración. En la mayoría de los enfermos, se rebajaron la toma de oxígeno y la toma del I^{131}, disminuyeron la ventilación total y la alveolar, aumentó la tensión del oxígeno alveolar y arterial y descendió la tensión del bióxido de carbono arterial. La mejoría clínica no se correlacionó siempre con los datos de laboratorio.

Resume

Résultats cliniques et physiologiques à la suite d'un traitement de l'insuffisance pulmonaire chronique par l'iode radio-actif (I^{131})

Vingt-trois patients atteints d'insuffisance pulmonaire sévère furent suivis durant deux ans et demi à trois ans et demi après un traitement par l'iode radio-actif. Les résultats cliniques et physiologiques sont présentés. L'amélioration clinique fut excellente chez 7 malades (30%), satisfaisante chez 2 (9%), moyenne chez 6 (26%) et insuffisante chez 8 (35%). L'étude de la fonction pulmonaire n'a révélé aucune amélioration significative en ce qui concerne les volumes pulmonaires ou le mécanisme de la respiration. Chez la majorité des patients la consommation d'oxygène et de I^{131} était réduite, la ventilation alvéolaire et totale diminuaient, la tension de l'oxygène artériel et alvéolaire augmentait et la tension artérielle du CO^2 diminuait. L'amélioration clinique ne coïncidait pas toujours avec les résultats du laboratoire.

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CHRONIC PULMONARY DISEASE DUE TO ATYPICAL MYCOBACTERIAL INFECTIONS[1, 2, 3]

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INTRODUCTION

The recognition of tuberculosis or tuberculosis-like disease in a wide variety of animals preceded the discovery of the tubercle bacillus by many years. Soon after the discovery of the tubercle bacillus it became evident that tuberculosis in various species was caused by organisms closely related to human tubercle bacilli but differing from them in specific and consistent ways.

The existence of acid-fast bacilli producing pulmonary disease in humans, but differing from *Mycobacterium tuberculosis* in cultural characteristics and in failure to produce progressive disease in the guinea pig, has been reported sporadically for many years, but with increasing frequency recently (1–13). These bacilli differ from certain other acid-fast organisms such as *M. ulcerans* and *M. balnei* (which have been reported to produce cutaneous lesions in humans occasionally), and also from *M. tuberculosis*, in their ability to grow at room temperature (13). They differ from *M. fortuitum*, another lesion-producing Mycobacterium which also grows at room temperature, as they differ from non-pathogenic saprophytic acid-fact bacilli in that they require at least six to twenty-one days to grow out on culture while the latter two grow in three days or less (13, 14). Three different types of atypical mycobacteria have been described and have been classified according to pigment production when exposed to light (15).

Review of the Literature

A review of the literature reveals that some of the cases of pulmonary disease due to these atypical acid-fast bacilli have been reported to have had previous isolations of *M. tuberculosis* with subsequent occurrence of atypical bacilli (8); many, however, have had no previous or concurrent association with tubercle bacilli (8, 11). In view of the fact that the majority have been reported to have no previous history of antituberculous drugs (8, 11), it does not appear likely that they represent tubercle bacilli altered by drug therapy. The roentgenographic, clinical, and pathologic picture produced by these organisms is generally described as indistinguishable from that of tuberculosis, although some pathologists have described an absence of typical Langhans' cells in human histology of this disease (5).

Tuberculin skin tests (standard PPD) of patients with atypical mycobacterial infection may be either positive or negative (11). Recent work utilizing purified protein derivative prepared from these atypical forms indicates that infection with atypical mycobacteria, especially the nonchromogenic variety,[4] is common in the southeastern part of the United States. Indications are that this may account for the high percentage of nonspecific reactions to standard PPD in this area (16). These organisms have been reported to produce cross-sensitization to tuberculin in the guinea pig (4).

Another characteristic of atypical acid-fast bacilli has been a high degree of resistance to antituberculous drugs (12, 17) and, with few exceptions, they have been reported to be resistant to most other antimicrobial agents. The antifungal drugs have also been investigated in this respect and there is evidence of resistance to these drugs (17). Other characteristics reported are a low percentage of "sputum conversion" and roentgenographic improvement on treatment as compared with tuberculosis (10–11), and the failure to find evidence of contact infection (11). Extrapulmonary disease involving variously the spleen, gastrointestinal tract, liver, adrenals, pancreas, skin, meninges, pleura, lymph nodes, joints, and bursa has been described as due to some of these organisms, as has miliary dissemination as well

[1] From the Southwest Florida Tuberculosis Hospital, Tampa, Florida.

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[3] Presented in part before the Medical Session, as part of *Section 4B*, at the annual meeting of the American Trudeau Society, Philadelphia, Pennsylvania, May 20, 1958.

[4] The term "nonchromogen" is used throughout this report to designate those organisms that are usually referred to as "nonphotochromogens" (Runyon Group III, Battey type).

(2, 3, 9, 10, 13, 18, 19). The occasional finding of these bacilli in persons with normal chest roentgenograms has been reported (8). Several cases of pulmonary infection associated with atypical mycobacteria occurring in relationship with achalasia have been reported (7, 20). These mycobacteria have been grown in tissue culture in HeLa cells, some showing branching forms similar to Nocardia (21, 22).

Antigen-antibody studies by the agar double-diffusion precipitin technique have shown that there is no single diffusible antigen common to all mycobacteria. These antigenic studies have shown no evidence of a relationship between mycobacteria and fungi, but have shown a close antigenic relationship between the atypical mycobacteria and virulent avian and mammalian tubercle bacilli. These atypical forms do not appear to be antigenically homogeneous. There was antigenic dissimilarity between atypical organisms and saprophytes in that atypical mycobacteria did not react with antisera prepared against saprophytic mycobacteria (23).

The world-wide distribution of these infections is indicated by numerous reports from this country and by the fact that the Sunmount Laboratory collection contains cultures from several foreign countries (24).

In November, 1955, the Florida State Board of Health, together with the Florida state tuberculosis hospitals, initiated a study of patients excreting atypical mycobacteria in their sputum. During a span of three years, 215 patient-strains have been identified among hospitalized patients and outpatients. One hundred and fifty-two of these cases have been studied in the four Florida state tuberculosis hospitals. Twelve (8 per cent) of these patients have been identified as excreting photochromogens; 112 (74 per cent), nonchromogens; and 28 (18 per cent), scotochromogens. This report, however, is an analysis of the first 100 consecutively studied patients from whom so-called atypical mycobacteria were isolated and studied in the Florida state tuberculosis hospitals.

Materials and Methods

Eighteen-hour sputum specimens were digested at 37° C. in 23 per cent (crystalline) trisodium phosphate. The specimens were centrifuged and smear and culture were prepared from the sediment. Smears were stained by the Ziehl-Neelsen method and each sediment was inoculated on two Löwenstein-Jensen medium slants. The slants were incubated at 37° C. and examined at weekly intervals for a maximum of eight weeks.

Cultures not readily identified as *M. tuberculosis* were selected for further study. Organisms were suspected of being atypical mycobacteria when smooth colonies appeared on primary cultures. Since *M. tuberculosis* will form smooth colonies on moist medium it is very important to employ medium with a dry surface. The catalase test was done in each instance (25). The neutral red reaction (26) was studied in the case of each strain, as was the presence or absence of cord formation (25). The rate of growth was determined by subculturing standardized subvisible suspensions of organisms on Löwenstein-Jensen medium at 37° C. and at room temperature. Additional Löwenstein-Jensen medium subcultures at 37° C., both in the presence and absence of light, were used to study pigmentation characteristics. Drug-susceptibility tests were performed on each culture. The size of the inoculum employed has been described previously by this laboratory (27). This size of inoculum was selected in order to obtain almost, but not quite, confluent growth on solid medium drug-free controls, thus permitting valid comparisons between amounts of growth on medium with and without added drugs. Tests performed on solid medium were read after three to four weeks of incubation. Tests performed in liquid medium were read at the end of seven and fourteen days of incubation. The following drugs were incorporated in Löwenstein-Jensen medium: streptomycin, PAS, isoniazid, cycloserine, thiazolsulfone (Promizole®), glucosulfone (Promin®), penicillin, and sulfisoxazole (Gantrisin®). The following drugs were incorporated in Difco oleic-acid agar: oleandomycin (Matromycin®), oxytetracycline (Terramycin®), tetracycline, phosphate buffered (Achromycin V®), thiocarbanidin, and kanamycin. Streptovaricin was prepared in Tween®-albumin liquid medium, and the tubes were inoculated within four hours after preparation.

Resected lung tissue was dissected aseptically along the bronchi. Separate instruments were used for each lesion. Portions of large lesions were cultured individually while clusters of small nodules were pooled. Löwenstein-Jensen medium was inoculated with material from each lesion, both before and after digestion in 23 per cent trisodium phosphate. Material from 7 lung specimens was inoculated into guinea pigs, 2 animals being used for each specimen. Cultures for fungi were made from pooled lesion material from 13 of the lung specimens.

Animal experiments: Two guinea pigs were inoculated subcutaneously with each patient-strain of atypical acid-fast organism. The inoculum for each animal consisted of 1 mg. of organisms from a ten- to fourteen-day-old Tween-albumin liquid medium culture. Skin tests employing 0.1 ml. of 5 per cent OT (5 mg.) were performed four and six weeks after inoculation. Animals were killed at the end of eight weeks. Smears and cultures were made from the site of inoculation, lymph nodes (if enlarged), spleen, liver, and lung.

Male dba mice were inoculated intravenously with each patient-strain of organism. Three mice were used for each experiment, and the inoculum consisted of 0.2 mg. of organisms from a ten- to fourteen-day-old Tween-albumin liquid medium culture. These animals were killed at the end of ten weeks. Smears and cultures were made from the spleen, liver, kidneys, and lungs of each mouse.

Observations

Bacteriologic

All of the 100 strains isolated differed from *M. tuberculosis* in a number of respects, and were characterized by a growth at room temperature and failure to form tightly woven cords in liquid medium. Ninety-six of the strains showed catalase activity greater than that of *M. tuberculosis*, whereas 4 showed equal catalase activity. The ability to bind neutral red was revealed by approximately one-half of the strains in each of the three groups of atypical organisms.

Classification of these 100 strains was based upon their ability to produce pigment when grown in continuous light and in total darkness. According to this classification, three major groups were distinguishable: the *photochromogenic* organisms that produced yellow pigment when grown in the presence of light, and white or buff-colored colonies when grown in the dark; the *nonchromogenic* organisms that produced white buff or yellowish pigment both in the light and in the dark; and the *scotochromogens*, a majority of which produced the same degree of yellow-orange pigment whether grown in the light or in the dark. Two strains produced yellow-orange pigment in the dark and red-orange pigment in the light.

The distribution of these strains, indicating that the majority fell in the nonchromogenic group, is shown in table 1. Of the 15 scotochromogenic strains, only 2 were of the common red-orange type, whereas 13 were of the less common and presumably more pathogenic yellow-orange variety.

As may be seen in table 2, the subculture growth rate of the photochromogens varied from six to twenty-eight days, that of the nonchromogens from six to forty-two days, and that of the scotochromogens from six to twenty-eight days. In most instances, the growth rate for each group at 37°C. was comparable with that of *M. tuberculosis*.

TABLE 1

Atypical Acid-fast Organisms Isolated from First 100 Patients in Study

Acid-fast Organism	Number of Patients
Photochromogen	7
Nonchromogen	78
Scotochromogen	15
Total	100

TABLE 2

Subculture Growth Rates of 100 Patient-Strains of Atypical Acid-Fast Organisms*

Days Before Visible Growth	Photochromogen		Nonchromogen		Scotochromogen	
	20° C.	37° C.	20° C.	37° C.	20° C	37° C.
<6	0	0	0	0	0	0
6–13	1	6	23	60	6	11
14–28	6	1	52	18	9	4
29–42	0	0	3	0	0	0
Totals	7	7	78	78	15	15

* Inoculum for each slant of Löwenstein-Jensen medium was 0.05 ml. of a 1:10 dilution of a faintly turbid suspension (subvisible suspension).

In 82 of the patients there was no proved isolation of true tubercle bacilli at any time, although in 21 of these there was an unsubstantiated history of the isolation of tubercle bacilli. Eighteen of the patients had bacteriologically proved tuberculosis.

In table 3 is listed the number of separate isolations of atypical organisms from the 61 patients having no history of *M. tuberculosis* isolations. The most striking feature of table 3 is the relatively large number of patients having multiple isolations of nonchromogens.

The time interval between the initial and most recent isolation of atypical organisms is shown in table 4. It may be noted that 9 patients excreted nonchromogens for more than twelve months. All patients received continuous chemotherapy. The repeated isolations over a long period of time in many cases are an indication of the degree of the drug resistance of most strains and emphasize the failure to respond to drugs.

Culture for other organisms was done in most cases, and in only one case was a significant pathogen, *Nocardia asteroides*, isolated. The

TABLE 3

NUMBER OF SEPARATE ISOLATIONS OF ATYPICAL ACID-FAST ORGANISMS FROM 61 PATIENTS WITH NO HISTORY OF *M. TUBERCULOSIS* ISOLATIONS

Organism	Number of Isolations Per Patient					
	1	2–5	6–10	11–20	21–30	>30
Photochromogen	1	2	1	1	0	0
Nonchromogen	2	12	12	15	4	1
Scotochromogen	3	4	1	2	0	0

TABLE 4

TIME INTERVAL BETWEEN INITIAL AND MOST RECENT ISOLATION OF ATYPICAL ACID-FAST ORGANISMS FROM 55 PATIENTS* WITH NO HISTORY OF *M. TUBERCULOSIS* ISOLATIONS

Organism	Interval in Months				
	<1	2–3	4–6	7–12	>12
Photochromogen	1	3	0	0	0
Nonchromogen	7	9	9	10	9
Scotochromogen	2	2	2	1	0

* The 6 patients with single isolations of atypical organisms are excluded from this table.

cultural characteristics of this patient's atypical mycobacteria were distinctly different from those of *Nocardia asteroides*.

It may be noted in table 5 that none of the group of patients with photochromogens and 18 per cent of the patients with nonchromogens had proved tuberculosis. In 9 of the nonchromogens only one isolation was obtained, and 6 of these patients had tuberculosis. Four of the 15 patients with scotochromogens also had tuberculosis; 3 of these were the yellow-orange type and one was red-orange. Seven patients with scotochromes had only single isolations, and 3 of these had tuberculosis. Consequently, 9, or over one-half, of the 17 single isolations of atypical mycobacteria occurred in the group having true tuberculosis.

Animal pathogenicity studies utilizing guinea pigs and mice revealed that none of these strains produced progressive disease in guinea pigs inoculated by the subcutaneous route. All of the photochromic strains, roughly one-half of the nonchromogenic strains, and none of the scotochromogenic strains, were pathogenic for mice.

Drug-susceptibility studies, including those on isoniazid, streptomycin, and PAS, were done in the case of 46 strains that could be tested before drug therapy, as is illustrated in table 6. Most of the strains of all three groups showed some degree of susceptibility to streptomycin in a concentration of 10 γ per ml. In most cases the susceptibility was only partial. In the case of nonchromogenic strains, only 2 of 35 showed complete susceptibility. Approximately one-half of the photochromes and scotochromes showed some susceptibility to isoniazid in a concentration of 1 γ per ml., although none of the photochromes was completely susceptible. Most of the nonchromogens were completely resistant to isoniazid and none showed complete susceptibility. Most of the photochromes and scotochromes showed some susceptibility to PAS in a concentration of 10 γ per ml., although for photochromes the susceptibility was never complete. Almost all of the nonchromogens were resistant to PAS. In the remaining patients who had received drug therapy prior to susceptibility studies, essentially the same pattern of drug susceptibility was noted.

Susceptibility studies were done in the case of streptovaricin, cycloserine, and viomycin for all 100 strains. All strains except 3 nonchromogens showed complete or partial susceptibility to 1 γ of streptovaricin, although in most cases the susceptibility was not complete. Most of the

TABLE 5

DISTRIBUTION BY STRAIN OF PATIENTS WITH PROVED *M. TUBERCULOSIS* INFECTIONS AND OF PATIENTS WITH A SINGLE ISOLATION OF ATYPICAL MYCOBACTERIA

Strain	Total Patients	Patients with Proved Tuberculosis	Single Isolation of Atypical Mycobacteria	
			No Proved Tuberculosis	Proved Tuberculosis
Photochromogen	7	0	1	0
Nonchromogen	78	14 (18%)	3	6
Scotochromogen				
Yellow-orange	13	3 (23%)	2	2
Red-orange	2	1 (50%)	2	1
Totals.....	100	18	8	9

TABLE 6

IN VITRO DRUG SUSCEPTIBILITIES OF 46 PATIENT-STRAINS OF ATYPICAL MYCOBACTERIA BEFORE THERAPY

Drug	Concentration in γ per Ml.	Photochromogen (5 Strains)			Nonchromogen (35 Strains)			Scotochromogen (6 Strains)		
		S*	PR†	R‡	S	PR	R	S	PR	R
Streptomycin	10	1	3	1	2	26	7	3	2	1
PAS	10	0	3	2	1	1	33	3	2	1
Isoniazid	1	0	3	2	0	5	30	2	1	3

* S = Susceptible
† PR = Partially resistant
‡ R = Resistant

TABLE 7

IN VITRO DRUG SUSCEPTIBILITIES OF 100 PATIENT-STRAINS OF ATYPICAL MYCOBACTERIA BEFORE THERAPY

Drug	Concentration in γ per Ml.	Photochromogen (7 Strains)			Nonchromogen (78 Strains)			Scotochromogen (15 Strains)		
		S*	PR	R	S	PR	R	S	PR	R
Cycloserine	20	6	1	0	29	39	10	9	4	2
Viomycin	10	1	1	5	8	16	54	3	1	11
Viomycin	100	6	1	0	45	26	7	13	2	0
Streptovaricin	1	1	6	0	36	39	3	2	13	0

* See footnotes to table 6.

strains showed some susceptibility to 20 γ of cycloserine, and in the case of 6 photochromes, 29 nonchromogens, and 9 scotochromogens, the susceptibility to this drug was complete. Less than one-half of the strains showed any susceptibility to 10 γ of viomycin, and in most cases such susceptibility as was demonstrated was only partial (table 7). Susceptibility studies in a small group of 20 cases revealed, in most instances, resistance to the broad-spectrum antimicrobials and sulfones. There was some degree of susceptibility to kanamycin and sulfisoxazole (Gantrisin®), as may be seen in table 8. Clinical use of these drugs has only recently been started.

Clinical

Infection with atypical mycobacteria is not rare in Florida. Two per cent of all of the patients admitted to Florida state tuberculosis hospitals show such infection. The distribution of the 100 patients by race and sex as compared with all patients hospitalized on January 1, 1956 is shown in table 9. That atypical mycobacterial infections due to nonchromogens occurred predominantly in the older white male may be seen in table 10. A majority of the patients were born and had lived in the southeastern United States. There was no apparent relationship with occupation or previous drug therapy. Most of the patients were from rural areas. In 28 instances there was a history of contact with *M. tuberculosis*, and the only previous contact with atypical infection was in the case of 2 stepbrothers who lived separately and had only casual community contact.

Tuberculin skin tests were done in the case of 95 family household contacts of this group, and 13 per cent were positive to intermediate-strength PPD. One hundred and thirty-three such contacts had chest roentgenograms, and 3 per cent of all of the cases showed changes compatible with tuberculosis.

In a majority of the cases the onset of illness had been insidious, but in 11 it had been acute. A few were asymptomatic, but the great majority had both general and local symptoms no different in kind from those associated with true tuberculosis. The physical findings were in no way differ-

TABLE 8

IN VITRO DRUG SUSCEPTIBILITIES OF 20 PATIENT-STRAINS OF ATYPICAL MYCOBACTERIA BEFORE THERAPY

Drug	Concentration in γ per Ml.	Photochromogen (2 Strains)			Nonchromogen (15 Strains)			Scotochromogen (3 Strains)		
		S*	PR	R	S	PR	R	S	PR	R
Amithiozone	10	0	2	0	0	11	4	0	1	2
Thiazolsulfone	20	0	2	0	0	2	13	0	1	2
Neomycin	10	0	0	2	1	4	10	0	0	3
Oxytetracycline	10	0	0	2	0	11	4	0	1	2
Tetracycline, phosphate-buffered	10	0	0	2	1	13	1	1	1	1
Oleandomycin	10	0	2	0	3	10	2	1	2	0
Chloramphenicol	10	0	0	2	0	4	11	0	1	2
Oleandomycin-tetracycline	10	0	2	0	5	10	0	1	2	0
Sulfisoxazole	20	2	0	0	6	7	2	2	1	0
Kanamycin	10	0	2	0	9	6	0	2	1	0
Thiocarbanidin	1	1	1	0	0	1	14	0	1	2

* See footnotes to table 6.

TABLE 9

SEX AND RACE OF FIRST 100 PATIENTS WITH ATYPICAL ACID-FAST BACILLI ISOLATIONS Florida Tuberculosis Hospitals 1955 to 1957

Sex and Race	Census of the Florida Tuberculosis Hospitals January 1, 1956		Patients with Atypical Organisms			
	Number	Per Cent	Nonchromogenic		Scotochromogenic	Photochromogenic
			Number	Per Cent	Number	Number
White male	593	36.8	52	66.7	8	6
White female	262	16.2	15	19.3	1	1
Nonwhite male	464	28.7	7	8.9	2	0
Nonwhite female	296	18.3	4	5.1	4	0
Totals	1,615	100.0	78	100.0	15	7

ent from those to be expected in patients with typical tuberculosis of equal extent. Infections due to the three types of atypical mycobacteria could not be distinguished from each other clinically.

The incidence of associated diseases which are predominantly pulmonary is shown in table 11. Such diseases as pulmonary emphysema and pulmonary tuberculosis were common in this series, suggesting some possible relationship between atypical mycobacterial infection and these diseases.

The majority of the patients who were skin tested with tuberculin were reactors. However, 11 of 61 patients with nonchromogens and one of 6 with photochromogens were nonreactors when skin tested with intermediate-strength PPD (0.0001 mg.) intradermally. All of the patients excreting scotochromogens were positive reactors, and the patients with proved tuberculosis were likewise all positive reactors.

The extent of disease as determined by an analysis of the admission chest roentgenograms in these cases is indicated in table 12. Roentgenographic abnormalities were analyzed in 82 of the patients from whom true tubercle bacilli were never isolated. Roentgenographically, the 3 atypical types could not be distinguished from each other or from tuberculosis. The extent of disease in the 64 patients with nonchromogens without coexisting tuberculosis was classified as moderately or far advanced in 90 per cent of the cases. Cavities were single or multiple, with thick or thin walls, and were present in 75 per cent of the cases. Approximately one-half of the cavities were greater than 4 cm. in diameter. The

predominant lesions were fibrotic or mixed in most cases, but in one-fourth of the cases the lesions appeared soft. Hilar or parenchymal calcification was present in more than one-half of the cases, and generalized emphysema was present in a significant proportion. In the 11 patients with scotochromes and 7 with photochromes without tuberculosis, cavitation occurred in 37 per cent and 72 per cent, respectively. Calcification was apparent in only 18 per cent of the patients with scotochromes, and in none of those with photochromes. Pleural thickening was seen occasionally, and in a recent case excreting nonchromogens, pleural effusion was visualized roentgenographically and proved by subsequent thoracentesis.

More recent analysis of 76 patients with disease due to nonchromogens, each with multiple isolations and without bacteriologic evidence of tuberculosis, revealed that serial chest roentgenograms demonstrated very slow resolution of the roentgenographic abnormalities in most cases. Only 20 per cent, most of whom had surgical resection, showed marked clearing. In 35 per cent of these cases there was either progression of the disease process or no change.

Thirty-six of the patients were examined bronchoscopically, and in only those patients with associated tuberculosis was significant bronchial abnormality noted. However, in a separate analysis of the first 100 patients with nonchromogens, 6 without coexisting tuberculosis had significant endobronchial disease. In 3 patients, one with scotochromogenic and 2 with nonchromogenic organisms, in none of whom *M. tuberculosis* had been isolated from the sputum, scalene node biopsy was done. Histologic changes were indistinguishable from tuberculosis. Cultures of the nodes were negative for acid-fast bacilli. There was no other evidence of extrapulmonary disease in the series.

Most of the patients were admitted with a diagnosis of tuberculosis, and the presence of atypical organisms was discovered later. Consequently, most patients were treated at first with 3 to 10 mg. of isoniazid per kg. and sodium PAS, 12 gm. daily. One-half of the patients treated with isoniazid and PAS attained noninfectious status. This was not necessarily because of the drugs. Of the group that failed to "convert" on isoniazid and PAS, about one-half "converted" when streptomycin was added. An additional small group "converted" when viomycin and cycloserine were added. It is to be noted that drug resistance to cycloserine developed rapidly, and some of the patients who "converted" were again discharging tubercle bacilli after a few months. Pyrazinamide and streptovaricin were

TABLE 10

AGE DISTRIBUTION OF 66* WHITE MALES ACCORDING TO ATYPICAL TYPE

Age Group	Photochromogen	Nonchromogen	Scotochromogen
24–44	1	12	1
45–54	4	11	3
55–74	0	25	4
More than 75	1	4	0
Totals	6	52	8

* 34 patients were either nonwhite or females.

TABLE 11

MOST COMMONLY ASSOCIATED DISEASES

	Photochromogen (7)	Nonchromogen (78)	Scotochromogen (15)
Pulmonary emphysema	1	42	3
Tuberculosis*	0	14	4
Asthma	0	7	1
Bronchitis	0	6	0
Bronchiectasis	0	3	0
Subtotal gastric resection	1	3	0
Bronchogenic carcinoma	0	2	0
Silicosis	0	2	1
Rheumatoid spondylitis	0	2	0
Diabetes	0	2	1
Duodenal ulcer	0	2	1
Sarcoidosis	0	1	0

* 0% of the patients with photochromogens, 18% of those with nonchromogens, and 27% with scotochromogens had proved tuberculosis.

TABLE 12

CLASSIFICATION OF EXTENT OF DISEASE BY ROENTGENOGRAPHY IN FIRST 100 PATIENTS

	Photochromogen	Nonchromogen	Scotochromogen
Minimal	0	9	4
Moderately advanced	5	30	4
Far advanced	2	39	7
Totals	7	78	15

TABLE 13

PRESENT STATUS OF FIRST 100 CASES

Strain	Pulmonary Disease Inactive or Inactive Except for Cavity	Pulmonary Disease Active	Patients Dead
Photochromogen..	3	4	0
Nonchromogen....	28	41	9
Scotochromogen...	11	3	1
Totals.....	42	48	10

complete failures, but were used in only a few cases. In only 2 patients excreting nonchromogens and 2 patients excreting scotochromogens was cavity closure achieved without surgery.

Twenty-five of the patients with cavitation were treated with major thoracic surgery: 24 had had resections and one had extraperiosteal plombage. In 10 of the 25, sputum was negative for mycobacteria preoperatively, and surgery was performed to eliminate cavities. The sputum of all of these patients remained negative for mycobacteria. Of the 15 patients excreting mycobacteria before surgery, 8 "converted" within a month of surgery and have remained negative. Seven patients, all excreting nonchromogens, continued to excrete mycobacteria after surgery, but in 2 the findings subsequently became negative. Most of them had had all significant disease resected, as determined by roentgenography. In one case the failure to "convert" was explained by a bronchopleural fistula. In most others there was no evidence of remaining significant disease, bronchiectasis, bronchial ulceration, bronchial fistula, or pleural fluid to explain the persistence of mycobacteria.

The incidence of surgical complications was 20 per cent, and there was one surgical death. One patient developed a bronchopleural fistula, and 4 required further surgery because of incomplete expansion of the lung. There was no apparent correlation between the type of Mycobacterium and the incidence of surgical complication. There was no correlation between the extent of surgery and the incidence of complications.[5]

[5] Further experience with resection in patients with nonchromogenic infections suggests that lobectomy is associated with fewer complications and is more likely to "convert" the sputum than segmental resection.

All 24 surgical specimens showed lesions grossly and histologically indistinguishable from tuberculosis. Cultures from the specimens were positive in 22 for organisms of the same kind as those found in the sputum preoperatively. Cultures for *M. tuberculosis* were negative in all cases. However, only 13 specimens were cultured for fungi; all were negative.

The present status of the 100 patients is shown in table 13. All of the patients with photochromic organisms are living, but 4 of this group still have active disease. Three patients with inactive disease had resectional surgery. One of the patients with scotochromogenic organisms died postoperatively, and 3 of the 14 living patients have active disease. Nine of the 78 patients with nonchromogenic organisms (11 per cent) are dead, and 41 of the 69 living patients (60 per cent) still have active disease.

In only one patient could death be attributed solely to an atypical mycobacterial infection. Four of the patients in whom death was due to progressive pulmonary disease had *M. tuberculosis* as well as atypical organisms in their sputum. In 4 patients death was due to malignancy. This included 2 patients with bronchogenic carcinoma, one with carcinoma of the colon, and another with carcinoma of the rectum. The remaining patient (with scotochromogenic organisms) died from postoperative staphylococcal pneumonia.

Autopsy was performed in 4 cases. In each case granulomatous pulmonary lesions were grossly and microscopically indistinguishable from tuberculosis. In each case in which mycobacteria were cultured from the pulmonary lesions, the organisms were identical with those present in the sputum before death.

DISCUSSION

In the past, most laboratories have designated acid-fast bacilli as either pathogens or saprophytes. Classification has otherwise been varied and subject to much dispute. Attempts at taxonomic identification have included animal pathogenicity studies, antigen-antibody reactions (23), chemical analysis of the bacilli (28), phage typing (29), tissue cultures (21, 22), and cultures on various media with variation in temperature, nutritional requirements, time of growth and, more recently, variation in pigment production on exposure to light. This latter method served as the basis of classification in this report.

Most cases of pulmonary disease reported in the literature which were due to atypical mycobacteria have been associated with the finding of chromogenic bacilli in the sputum. In many of the earlier reports, scotochromes were not well differentiated from photochromes. Most recent reports have emphasized the fact that the photochromes are a closely related group of organisms. Animal studies have revealed pathogenicity for the mouse, monkey, and hamster. Skin testing of patients with disease due to these bacilli has revealed that such persons develop reactions to both atypical photochromic PPD and standard PPD, and these reactions are of comparable size (16). Other investigators have shown that there is a closer relationship between the photochromic organisms and tubercle bacilli than between the other atypical organisms and tubercle bacilli (17). Cases of pulmonary disease due to photochromogenic organisms have been described over a wide area of the world. The greatest number reported to date, however, has been found in the northern central states of the United States (30). The present series of photochromes is too small to provide definite conclusions.

Scotochromes, like photochromes, vary in rate of growth. In contrast with photochromes, however, scotochromes vary in production of pigment and, more often than otherwise, occur in association with other diseases such as silicosis and emphysema. There has not been the certainty that they are causative of disease as has been the case with the photochromes. They do not produce disease in any laboratory animal. In the present series of 15 cases, solitary isolations were common, as was coexistence with other diseases. Runyon has made a distinction between the yellow-orange and the red-orange scotochromogens. The former produce the same degree of pigment whether grown in the dark or in the presence of light. On the other hand, the red-orange scotochromogens are yellow to orange when grown in the dark, and red-orange when grown in the light. These red-orange scotochromogens are not considered to be independent pulmonary pathogens; however, they may occur in cervical adenitis or other superficial infections. The fact that these strains may be isolated from distilled water and other nonclinical material suggests that as a group they are saprophytes (31). Although in some of the present cases the scotochromogen isolated was not considered to be significant as a cause of disease, in others there appeared to be little doubt that it was the causative organism since it was isolated repeatedly, both from sputum and from surgical specimens, to the exclusion of other causative organisms. The group of scotochromogens was not as distinct in its drug-susceptibility patterns as some of the other atypical strains, showing a variation in susceptibility to streptomycin, isoniazid, and PAS. Pathologic study has shown gross and microscopic changes identical with tuberculosis.

In contrast to the other two groups, relatively little has been written about the group of nonchromogens which is the most numerically important group in our series, consisting of more than 100 strains isolated since this study began (although this report concerns an analysis of only the first 78). The preponderance of this type of atypical organism in the Florida hospitals is consistent with the fact that most of the cases reported have been isolated in the southeastern part of the United States, as would be expected from the result of skin testing with specific PPD prepared from nonchromogens (16). The epidemiologic studies have revealed that, as has been reported previously, the incidence of positive tuberculin skin tests and abnormal roentgenographic findings in patient contacts has been lower than would be expected in the contacts of patients with tuberculosis and, in general, there has been no evidence of contagiousness to date (11). However, these studies are incomplete and so far have shown no pattern that would suggest the method of infection. Atypical PPD has not as yet been used for skin testing of contacts. In this series, predominantly white patients, especially white males, have been infected. Infection in Negroes has been rare, and 6 of 17 such infections have been due to scotochromogens.

The bacteriologic and animal observations in the present study suggest that this is not a distinct species or as closely related a group as are the photochromogens. This observation was based upon cultural variations between strains and the fact that they vary in pathogenicity for mice. The drug-susceptibility studies have been rather consistent in showing resistance to isoniazid and PAS and partial-to-complete susceptibility to streptomycin, cycloserine, viomycin, streptovaricin, kanamycin, and sulfisoxazole.

This group of mycobacteria is regarded as less pathogenic than tubercle bacilli, but it is be-

believed that they may occasionally cause disease in an otherwise healthy person, and not infrequently cause disease in lungs, the site of previous pathology. A striking feature of this study was the relationship between infection with nonchromogens and other diseases, particularly tuberculosis and emphysema. The writers have been inclined to dismiss as unimportant the finding of nonchromogens on only one isolation, especially if other causative organisms can be found. In most of the cases, however, multiple isolations were obtained, and in many there was no other apparent cause for the pulmonary disease except these microorganisms which, in such instances, appeared to be the true etiologic agent.

The present cases have been analyzed in a search for some characteristic that would distinguish disease produced by atypical mycobacteria from tuberculosis, but no distinguishing clinical feature could be found. Similarly, the diseases caused by atypical mycobacteria cannot be distinguished from each other by clinical, roentgenographic, or pathologic features.

Recently, microbiologists have postulated a spectrum of mycobacteria with tubercle bacilli at one extreme of great pathogenicity and the saprophytes at the other extreme. They place the group of photochromogens next to tubercle bacilli, the group of nonchromogens adjacent to this, and the group of scotochromogens next to the saprophytes (32).

Infections with the nonchromogens are relatively resistant to treatment when compared with the treatment of tuberculosis with antimicrobials. Drug therapy was frequently ineffective, and surgical treatment, although productive of greater success, also met with a significant number of failures from the standpoint of "sputum conversion." At the present time, the writers believe that treatment should consist of the following: (*a*) isolation from patients with active tuberculosis for mutual protection from possible cross-infection; (*b*) rest, which could perhaps be used with profit to a greater degree than in this series; (*c*) isoniazid, mainly because of the frequency of concomitant tuberculosis; (*d*) other antibacterial drugs, particularly streptomycin, cycloserine, viomycin, possibly streptovaricin, sulfisoxazole, thiocarbanidin, and kanamycin, as indicated by the susceptibility of the strain recovered from the patient. A combination of at least two of the drugs to which susceptibility is present should be used; (*e*) surgical resection in the absence of rather prompt "sputum conversion" under the protection of one or more drugs to which the patient's organisms have been shown to be susceptible.

Summary

The patient population of the Florida state tuberculosis hospitals has been systematically surveyed by careful study of sputum cultures for the occurrence of atypical mycobacteria, and such infections were discovered in 2 per cent of the patients so studied. Of the 100 atypical mycobacterial strains reported in this paper, 7 per cent were photochromogens, 15 per cent were scotochromogens, and the remaining 78 per cent were nonchromogens. Disease caused by these organisms occurred predominantly in elderly white males with diseased lungs who lived in rural areas.

In 74 per cent of these patients, atypical mycobacteria appeared to be the cause of pulmonary disease, since 26 per cent had either proved tuberculosis or single isolations of atypical mycobacteria. No evidence of contagiousness of atypical mycobacterial infection was found, but transmissibility from man to man cannot be excluded. Pulmonary disease produced by atypical mycobacteria was clinically, pathologically, and roentgenographically indistinguishable from tuberculosis, and can be properly diagnosed only by careful bacteriologic study. Treatment with a sanatorium regimen consisting of isoniazid and a variety of other antibacterial drugs, as well as resectional surgery when feasible, has been relatively unsatisfactory. Ten per cent of the patients are dead (only one from progressive atypical mycobacterial infection alone), and 48 per cent of the living patients still show mycobacteria in their sputum, a total rate of therapeutic failure of 58 per cent. Treatment should consist of rest, antimicrobial therapy with isoniazid and two or more drugs to which the patient's organisms are susceptible, and surgery in suitable cases to remove cavity or significant residual foci of disease.

Sumario

Enfermedad Pulmonar Crónica debida a Atípicas Infecciones Micobacterianas

Los enfermos de los hospitales de tuberculosis del Estado de Florida fueron objeto de una indagación sistemática en un estudio cuidadoso de cultivos del esputo en busca de micobacterias

atípicas, descubriéndose éstas en 2 por ciento de los enfermos estudiados. De las 100 cepas micobacterianas atípicas mencionadas en este trabajo, 7 por ciento eran fotocromógenas, 15 por ciento estotocromógenas y las 78 por ciento restantes no eran cromógenas. La enfermedad provocada por estos microbios predominó en los sujetos blancos de adad avanzada que tenían los pulmones afectados y vivían en zonas rurales.

En 74 por ciento de estos sujetos, micobacterias atípicas parecían ser la causa de enfermedad pulmonar, dado que 26 por ciento tenían ya tuberculosis comprobada o aislamientos aislados de micobacterias atípicas. No se descubrieron signos de contagiosidad de la infección micobacteriana atípica, pero no cabe excluir la transmisibilidad de persona a persona. La enfermedad pulmonar producida por las micobacterias atípicas era clínica, patológica y radiográficamente indistinguible de la tuberculosis y no puede diagnosticarse debidamente más que con el cuidadoso estudio bacteriológico. El tratamiento con un régimen sanatorial que comprende isoniacida y otras varias drogas antibacterianas, y con la resección quirúrgica cuando es factible, ha resultado relativamente poco satisfactorio. Diez por ciento de los enfermos han fallecido (solamente uno de infección micobacteriana atípica evolutiva por sí sola) y 48 por ciento de los enfermos vivos todavían muestran micobacterias en el esputo, o sea un coeficiente total de 58 por ciento de fracasos terapéuticos. El tratamiento debe consistir en el descanso, terapéutica antimicrobiana con isoniacida y dos o más medicamentos a los que sean susceptibles los microbios del enfermo y la cirugía en los casos apropiados para eliminar las cavernas o los importantes focos residuales de la enfermedad.

Resume

Maladies pulmonaires chroniques dues à des infections mycobactériennes atypiques

La population hospitalisée dans les hôpitaux de l'Etat de Floride a été systématiquement observée à l'aide d'une minutieuse culture des crachats pour déceler la présence de mycobactéries atypiques. Ces infections ont été décelées chez 2% des patients ainsi étudiés. Parmi les 100 souches mycobactériennes atypiques mentionnées dans cet article, 7% était des photochromogènes, 15% des scotochromogènes et les 78% restants des non chromogènes. La maladie causée par ces organismes survenait de façon prédominante chez les hommes âgés, de race blanche, dont les poumons étaient altérés et qui résidaient dans les régions rurales.

Chez 74% de ces malades, les mycobactéries atypiques paraissaient être la cause de la pneumopathie, car chez 26% d'entre eux il y avait eu une tuberculose apparente ou des mycobactéries atypiques avaient été isolées. Aucune preuve de contagiosité de l'infection mycobactérienne atypique n'a été découverte, mais la contamination d'homme à homme ne peut être exclue. La maladie pulmonaire produite par les mycobactéries atypiques ne pouvait être distinguée de la tuberculose soit cliniquement, pathologiquement ou radiologiquement, seule une étude bactériologique minutieuse permettait le diagnostic. Le traitement avec régime sanatorial, qui consistait en isoniazide et en divers autres agents antibactériens plus la chirurgie d'exérèse, quand celle-ci était practicable, s'est montré relativement peu satisfaisant. Dix pour cent des malades sont morts, (1 d'entre eux uniquement par infection mycobactérienne atypique progressive) et 48% des malades vivants ont encore des mycobactéries dans l'expectoration, soit un taux d'échecs thérapeutiques de 58%. Le traitement doit consister en repos, traitement antibactérien avec isoniazide associée à deux ou plusieurs drogues auxquelles les germes du malade sont sensibles et, dans les cas adéquats, intervention chirurgicale pour réséquer la caverne ou tout autre foyer pathologique résiduel significatif.

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TUBERCULOSIS AMONG THE NAVAJO[1, 2, 3]

Research in Cross-cultural Technologic Development in Health

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Introduction

In July, 1955 the Department of Public Health and Preventive Medicine, Cornell University Medical College, initiated a field health research project among the Navajo Indians in Arizona. The project, which is supported by the Navajo Tribe and the United States Public Health Service, developed from a Cornell research project in tuberculosis that had been started in January, 1952.

The Navajos, now numbering 85,000, are the largest Indian tribe in the United States. They still retain their own distinctive culture, language, and religion. In many respects the Navajos have political, social, economic, and health problems similar to those of people from the so-called economically underdeveloped areas of the world. In matters of health, tuberculosis has been a major medical problem among the Navajo people. However, during the past seven years, intensive efforts by the Health Branch of the Bureau of Indian Affairs, the Division of Indian Health of the United States Public Health Service,[4] Cornell University Medical College, and the Navajo Tribe have had salutary effects on tuberculosis morbidity and mortality.

Although the cross-cultural efforts of the Cornell field project were initially motivated by an attempt to attack the problem of tuberculosis, they were soon extended to embrace many additional research problems, and ultimately included a study of all aspects of health and disease in this Navajo community.

Navajo Concepts of Tuberculosis and Its Relation to Environment

The Navajos call tuberculosis a "fading away of the heart." Traditionally they say that it is caused by improperly performed wind chants or contact with an object, such as a tree struck by lightning. Although death is considered "contagious" by the Navajo, tuberculosis is not regarded as a contagious disease.

The Navajos believe that tuberculosis may be cured by certain ceremonies known to only a few medicine men. However, even these medicine men or singers who adhere to their ancient rituals now acknowledge the superiority of modern medicine in treating tuberculosis.

But the fact that a Navajo has accepted modern treatment of tuberculosis does not necessarily indicate that he has abandoned his traditional beliefs. This is illustrated with the following experience.

In 1953, Mr. Manuelito Begay, a respected medicine man and Tribal Councilman, attended a health education program at a Reservation tuberculosis sanatorium. The cause of tuberculosis was demonstrated as the tubercle bacilli which he was shown under the microscope. Peering into the eyepiece of the instrument, Mr. Begay exclaimed: "Today I have met the enemy of the Navajo people!" However, our conviction that this Navajo medicine man had been dramatically converted to the germ theory of tuberculosis was soon dispelled. For, several months later, when the Cornell studies were being presented to the Navajo Tribal Council, the convert arose to speak. At one point he gestured to the Cornell physicians, and the Council responded with peals of laughter. We later learned from the interpreter that Mr. Begay had generally praised our work and had said the "white doctors" and the Navajo medicine men were working for the same goal—good health—even though they might have their differences in concepts. The laughter had been evoked by his

[1] From the Department of Public Health and Preventive Medicine, Cornell University Medical College, New York, New York.

[2] This work has had the generous support of the Navajo Tribe; the Division of Indian Health; The National Institutes of Health of the U. S. Public Health Service; and the Russell Sage Foundation.

[3] Speech given at the annual meeting of the New York Tuberculosis and Health Association, New York, New York, on April 8, 1959.

[4] On July 1, 1955, the responsibility for the health of the U. S. Indians was transferred from the Bureau of Indian Affairs, Department of the Interior, to the U. S. Public Health Service, Department of Health, Education, and Welfare.

statement:

"These doctors tell me tuberculosis is inflicted by a person coughing in your face. Right away I disagree with it. A person should not be that weak to be susceptible to a man's cough. We have a definite point in mind and know how a man gets to be afflicted with tuberculosis. One is the ceremony about the wind chant. If something goes wrong with that it is tuberculosis; and if lightning strikes you, tuberculosis is the result."

Our wise elder was trying to teach us the folly of spending too much time on conceptual differences to the neglect of practical measures.

Another example points out the treacherous waters on which one embarks in attempting to simplify scientific medicine to perceptive but nonliterate people.

Several years ago during a field survey of the tuberculosis problem in the Reservation, a group of Cornell physicians stopped at the hogan of an elderly Navajo woman. The doctors told her about the general purpose of their visit. When she asked these doctors what was the cause of tuberculosis, they answered that it was something in the spit from a person sick with the disease. Upon hearing this, the Navajo woman shook with laughter and pointed to an old scraggly rooster that was strutting around the hogan. "In that case," she said, "that old rooster should have been dead from tuberculosis a long time ago—he's always pecking away at the spit all around here!"

Long ago, the Navajos empirically and intuitively associated disease with their social environment. This was forcefully expressed by Mrs. Annie Wauneka, the Chairman of the Navajo Tribal Council Health Committee.

"Mention has been made of the 1930 Federal program of reducing our sheep herds. This was done without the consultation of the Navajo Tribe. I guess we had some diseases like tuberculosis before that. But it seems to me our basic food was taken away from us at that time, all of a sudden. This has exposed us to such diseases as tuberculosis. We have found out that from then on the Navajos seemed to have insufficient food and seemed to find that they had tuberculosis and other diseases. Before that, we had sufficient food, and it seems to me the diseases weren't as common as they are today."

The entrenched beliefs in the traditional concepts of etiology and treatment of tuberculosis still persist and exert a real influence on the Navajo patients and the effectiveness of physicians in treating them. The following anecdote will illustrate the point.

In 1954 a group of 50 Navajos were hospitalized in an off-Reservation sanatorium in Tucson, Arizona, some 200 miles away. During a violent summer storm, lightning struck a nearby palm tree. Shortly thereafter, these patients became restive, and several decided to leave the hospital despite all attempts to reassure them. A hurried telephone call was made to the Reservation health office. After getting advice from the Reservation physicians concerning the attitude about Navajo lightning taboos, it was decided to call a medicine man to perform a ritual neutralizing the evil produced by the lightning. A Navajo medicine man from Fort Defiance was flown to Tucson. A ceremony was performed with the ritual chanted over an "intercom" system. Anxiety was relieved and peace of mind restored.

Families also have demonstrated changing attitudes and behavior patterns to modern medicine. Among other things, this influences the decision whether a patient will remain in the hospital or go home for a Navajo ceremony.

On many occasions, a patient's relatives would ask for his clothing as they left after a visit to the sanatorium. They would explain that they wanted to launder it in preparation for the patient's discharge. Careful inquiry, however, elicited the truth. They were not interested in washing the garments but in using them as a proxy for the patient in a "blessing or healing" ceremony.

The Reservation-Wide Tuberculosis Program

Next, let us turn to the results of the tuberculosis treatment program on the Navajo Reservation. To understand what has happened, it is pertinent to review the tuberculosis problem before the expanded Federal program became available in the fall of 1952.

In 1950 tuberculosis was one of the leading causes of death on the Navajo Reservation. There was only one tuberculosis hospital with one hundred beds for the entire Tribe. There were so many tuberculous patients who could not be hospitalized that case-finding programs were abandoned. Miliary and meningeal tuberculosis were commonplace even in adults. Estimates of active disease among Navajos ranged between

2 and 3 per cent, and infection rates measured by positive tuberculin reactions were 50 to 60 per cent among school children in the six- to ten-year age bracket. This was the situation until 1952, when two major changes occurred.

(*1*) A government program of contracting with off-reservation sanatoriums to care for patients with active infectious tuberculosis was established.

(*2*) The new and potent antituberculous drug, isoniazid, became available.

The sole facility for the care of tuberculous patients on the Reservation—the 100-bed tuberculosis service at Fort Defiance, Arizona—was made the focal point of the program. Arrangements were made with Cornell University Medical College to supply the professional direction of this facility, and the physicians in charge held University appointments as well as their appointments within the Government service.[5] The facility was organized to serve as both a University research unit for the study of the chemotherapy of tuberculosis and the principal evaluation center through which the patients were transferred from the field to the off-reservation hospital services.

The impact of this "crash" program in hospitalization combined with the widespread use of isoniazid was soon demonstrated (1). By 1957 there was a 40 per cent drop in tuberculosis mortality. Contrary to some medical opinion, Navajos responded like their "Anglo" neighbors to adequate medical and surgical treatment. During the five-year period from 1952 to 1957, approximately 1,000 patients were admitted to the tuberculosis hospitals—or virtually half the estimated active cases. At the five-year evaluation point, 79 per cent of the 500 patients discharged from these hospitals were considered to have received maximal hospital benefit. Of the 21 per cent who left the hospital before discharge was considered advisable, many were able to benefit from chemotherapy on an outpatient basis (1).

Over the past seven years, the situation has improved enormously. The miliary-meningeal problem is vanishing; no patient with active tuberculosis is required to wait for a hospital bed; and the tuberculin reactor rate among the six- to ten-year olds in the Reservation schools has fallen to levels of 20 per cent.

A review of the tuberculosis case register for 1958 among all Navajos shows that only 242 patients are now hospitalized, only 15 died of tuberculosis last year, and 171 were discharged from the sanatoriums and continue on chemotherapy (2).

Thus, it was soon apparent that the extensive tuberculosis problem among the Navajos was yielding to the health measures instituted by the Government and the Cornell University investigators.

As welcome as these hospital successes were, it was obvious to the United States Public Health Service that these gains in tuberculosis control might only be temporary unless some of the health measures could be extended into the home. In effect the visible top of the tuberculous "iceberg" had been lopped off, leaving a real need to determine how much was still submerged. Thus, if these gains were to be maintained, a practical field program had to be developed.

But, what kind of field program was feasible in the face of such conceptual differences as have been described? Research was obviously needed to obtain information on such questions, and so the Cornell-Many Farms Project was initiated as a joint enterprise of the Navajo Tribe, the United States Public Health Service, and the University. Although the problem of tuberculosis was high on the research agenda, it is only one of many medical studies included in this field health research program. However, it is only the results of the tuberculosis studies that concern us today.

Cornell Field Studies of the Navajo Tuberculosis Problem

The field health research project, which was actually started in May, 1956, has been in continuous operation in an 800-square mile region in the north-central part of the Navajo Indian Reservation known as the Many Farms-Rough Rock-Valley Store area. Within this district live 2,000 Navajos, about half of them in scattered camps in a dry sandy valley region; the others, in the mountainous and canyon country to the West. The clinic at Many Farms is the headquarters for the Project. To date, 80 per cent

[5] In addition to the speaker, the physicians who served successively in this capacity (1952–1959) are: Drs. Avrum Organick, Clarence Jordahl, and Roger Des Prez. A number of fourth-year Cornell medical students likewise have given considerable assistance to the enterprise.

of the Navajos in the area have been seen in the clinic, where between 400 and 600 visits are made per month. Of the 1,600 Navajos registered in the Clinic, it has been possible to obtain complete histories and physical examinations on 75 per cent, roentgenograms of the chest in 80 per cent, and tuberculin tests on 62 per cent of the 834 children less than fifteen years of age.

During the period January, 1957, to May, 1958, the prevalence of tuberculosis in a subsection of the Project area was carefully studied (3).

The senior investigator in this study was Dr. Abdel Omran from the faculty of the University of Cairo, Egypt. The results of this epidemiologic study are incorporated in the dissertation that Dr. Omran wrote in partial fulfillment of the requirements for the doctorate at the Columbia School of Public Health, and will also appear in the scientific periodical literature (3).

The purpose of Dr. Omran's research was to determine the value of utilizing school and preschool children as "index cases" for tuberculous disease in the community.

Because a complete health evaluation of the Navajo community was part of the general field health program, tuberculin tests as well as roentgenograms could be obtained on both the tuberculin reactors, the nonreactors, and their household associates. The unique feature of this study was that it was possible to compare within the same community the results of a case-finding program obtained by the standard mass roentgenographic method with those obtained by the tuberculin technique. In effect, then, the community acted as its own control. To our knowledge this is the only study in which such an internal comparison has been reported. A 400-square mile area containing 923 native Navajos living in scattered camps was selected. Persons less than thirty-five years of age constituted 76.6 per cent of the study population, a proportion that parallels the tribal population as a whole. Chest films were available on 90 per cent of the total study group. Of the 520 children less than twelve years of age, 470, or 90.3 per cent, had complete clinical examinations, including chest films. Tuberculin testing in this group was complete in 376 (72.3 per cent), 83 of whom had positive reactions, accounting for an infection rate of 22 per cent.

Of the 923 native Navajos in the Many Farms area, 81 had pulmonary tuberculosis and 7 had extrapulmonary tuberculosis, 5 with, and 2 without, associated pulmonary tuberculosis. Thus, of 81 persons who had pulmonary tuberculosis: 43 had arrested reinfection tuberculosis; 20 had active reinfection tuberculosis; 13 had active primary tuberculosis; 5 had chronic pulmonary disease, most likely of tuberculous etiology.

On the basis of these data, the prevalence rate of tuberculosis among the 923 Navajos was 9 per cent for all forms of tuberculosis, 8.6 per cent for pulmonary tuberculosis (not including healed primary tuberculosis), 7 per cent for reinfection pulmonary tuberculosis clinically significant by roentgenographic examination, and 2.1 per cent for active reinfection pulmonary tuberculosis.

The tuberculin testing of preschool and school children led to follow-up studies of household associates, which in this community includes about half the population. These studies uncovered 81.6 per cent of all forms of tuberculosis, 78.9 per cent of reinfection pulmonary tuberculosis, and 75 per cent of active reinfection pulmonary tuberculosis diagnosed by roentgenographic methods.

The relative prevalence of tuberculosis among the different households or camps was striking. The morbidity rate of tuberculosis in all forms was 135 per 1,000 camps with tuberculin-positive children (school and/or preschool), and only 27 per 1,000 in camps where all children were tuberculin negative. The comparative rates of active reinfection pulmonary tuberculosis were 25 per 1,000 and 7 per 1,000, respectively.

On the basis of this study, it was concluded that tuberculin testing of school and *preschool* children was a highly effective "index" method for determining the presence of tuberculosis in a community. It is, of course, recognized that the school children are a captive population, and hence easily accessible for tuberculin-testing programs. Preschool children, on the other hand, may be particularly difficult to reach, especially in countries with underdeveloped resources. However, the additional effort required to screen preschool children is amply rewarded by increasing the yield of tuberculous foci as well as by uncovering other health problems.

Tuberculin surveys of the Project school population have been conducted periodically since May, 1956. Results of these studies have revealed: (*1*) a decrease in the positive tuberculin skin reaction from a high in 1956 of 33.3 per cent in six- to ten-year olds, to 22.2 per cent in May,

1958. (*2*) Only one child has been definitely observed to convert from a negative to a positive tuberculin skin reaction. Thus, of the 150 children negative to intermediate-strength PPD tuberculin in each of the two school years where both fall and spring surveys were conducted, one convertor in 300 occurred. This represents a conversion rate of 0.3 per cent. (*3*) The admission or beginner (age five and a half to six years) group of school children in the Project area had a 24 per cent tuberculin reactor prevalence in 1956, but by 1958 the figure had dwindled to 16.3 per cent. A detailed scrutiny of these 8 positive tuberculin skin reactors in 1958 revealed that only 2 had not previously been identified as positive by our staff. Three had already been discovered in a preschool testing program, and 3 were discharged sanatorium patients.

The tuberculin testing surveys clearly point out the high prevalence of tuberculous infection among the Project school children. Similar studies in other Reservation areas confirm the fact that this high prevalence obtains throughout the Tribe (4, 5). Comparison with children in the same age brackets in other communities in the United States indicates that the Navajo tuberculin positive reactor rate is at least ten times greater.

It would seem, however, that the massive hospitalization program for patients with active tuberculosis has already largely wiped out the reservoir of active infectious tuberculosis. Evidence for this is found in these facts: the tuberculin rate is falling, especially in the preschool group; only one tuberculin convertor was found in a two-year observation period; and active clinical forms of tuberculosis, including miliary and meningeal, have practically disappeared.

It should be recalled that the five and a half and six year olds who entered the Many Farms school last fall were born about the time when the government hospital program was initiated and isoniazid became generally available in the treatment of tuberculosis. The full effects of hospitalization and chemotherapy on entering school children may not be seen until around 1962 or 1963.

After the children who were tuberculin reactors were identified, prophylactic isoniazid therapy was instituted for a period of twelve to eighteen months. Approximately 120 children, most of them in the three primary grades age six to ten years, have completed this chemotherapy program.

About 40 patients were treated with isoniazid while ambulatory because of the presence of active or questionably active pulmonary disease. At least 18 of these patients had been started on therapy during hospitalization and were continuing to receive tuberculosis chemotherapy following discharge from the hospital. Thus, a total of 160 patients have been carried on a self-administered isoniazid treatment program during the past three years.

Most of the patients apparently took the isoniazid as prescribed, although it was obviously not possible to determine this with certainty in all cases. It is of interest in this connection to review the experience in the cooperative United States Public Health Service study (6) conducted by Ferebee and her associates. These investigators compared two groups of patients with primary tuberculosis, one treated with placebo, the other with isoniazid. They showed that 80 per cent fewer complications were seen in the isoniazid-treated group. Although it was not possible to determine exactly how the individual patients behaved with respect to faithfulness in taking the drug, in the group as a whole, sufficient drug was obviously taken to show a substantial protective effect.

Of the 2,000 Navajos in the Project district, 59 have actually been hospitalized for active tuberculous disease. Of these, 24 (40 per cent) were males and 35 (60 per cent) were females. This represents 3 per cent of the community who have required sanatorium care. It is not surprising to find that 90 per cent of these hospitalizations occurred after 1951. Fifty-one patients have already been discharged from the hospitals, 8 are now being treated in various sanatoriums, and only 2 have flatly refused treatment in a hospital.

The clinical forms of tuberculosis in those who were hospitalized were as follows: reinfection pulmonary tuberculosis, 48; progressive primary pulmonary tuberculosis, 5; and extrapulmonary tuberculosis, 6.

Five patients with reinfection pulmonary tuberculosis have associated extrapulmonary disease: 2 patients with meningitis, 2 with renal disease, and one with disease of the bone.

The six extrapulmonary forms of tuberculosis included meningitis in 2 patients, bone tuberculosis in 2, renal disease in one, and lymph node involvement in one patient. All 4 patients with

meningitis were successfully treated despite the fact that 3 of them were adults.

If this community picture of the disease is representative of the Tribe as a whole—and we believe it is representative—the magnitude of the tuberculosis problem is established. It should be emphasized, however, that only 2 Navajos in the district actually refused recommended sanatorium treatment. These individuals are excreting tubercle bacilli in their sputum. One has already infected all of his five children, each of whom is now on isoniazid therapy. The other, a middle-aged woman, is not living with any children or tuberculin-negative adults. The contacts of these patients are under careful surveillance.

Finally, in this connection, the effectiveness of the hospital and chemotherapy programs can be further appreciated when it is observed that no person in the community died of tuberculosis during the three-year period that the Many Farms project has been in full operation.

When we had established the high prevalence of tuberculosis in this Navajo community, a BCG vaccination program for newborns was considered. Since half the children in the Project area were born in hospitals where BCG vaccination is offered, this indicated that our field staff would have to vaccinate approximately 50 babies born each year at home.

About the time we were ready to initiate our BCG immunization program, the results of additional tuberculin testing and clinical studies became available and revealed these facts:

(*1*) The school age group appears to face little or no risk, since only one tuberculin convertor was established in two years.

(*2*) The beginners in the Project schools showed a remarkable drop in tuberculin reactions from 24 per cent in 1956 to 16.3 per cent in 1958. The tuberculin tests of the preschool children provide a sensitive index of what is going on in the community with respect to active tuberculosis, leading to active foci of infection.

(*3*) Absence of miliary-meningeal disease in infants and young children seems to be evidence that tuberculosis has been controlled in this community. On the basis of this information, we decided not to administer BCG to the newborns. For the present, we still recommend BCG for those infants and children exposed to active tuberculous contacts who refuse hospitalization. Whether a BCG program for older children or adolescents is indicated is not yet clear, but may well prove to be indicated on the basis of further study.

* * *

It has been shown that, despite firmly held traditional beliefs in the etiology of tuberculosis and in treatment by native healing ceremonies, modern medicine for the management of this disease has been accepted on the Navajo Indian Reservation.

Since the advent of an expanded hospitalization and chemotherapy program, the tuberculosis mortality and morbidity have tumbled steadily downward. Nevertheless, because tuberculosis is so chronic, the presence of 7 per cent of clinically significant active or inactive disease on the basis of chest films still marks the Navajo as a tremendously high prevalence area. Detailed studies in the Project community further confirm the fact that at least 3 per cent of the population has had recently active tuberculous disease. These findings stand in sharp contrast to a contemporary tuberculosis prevalence study (7) in Annandale, Scotland, where clinically significant tuberculosis was found in only 0.5 per cent of this modern community.

The rapidly changing picture of clinical disease and tuberculin reactor rate among the Project Navajo, which appears to be a Reservation-wide phenomenon, suggests that the intensive hospital and chemotherapy measures may bring the tuberculosis problem within control in the near future. It can also be said that we do require an imaginative field program for *continued* tuberculosis control.

To achieve and maintain the control of tuberculosis on the Navajo Reservation, the medical staff should be able to keep a constant check on approximately 2,000 patients with active or recently active tuberculosis. Many of this group have recently been discharged from off-Reservation sanatoriums with instructions to continue ambulatory chemotherapy and periodic clinical re-examinations. There may be another 4,000 Navajos with clinically significant inactive pulmonary tuberculous lesions who might well be added to the reservoir of infectious tuberculous foci if and when the lesions break down. Thus, in addition to the 2,000 or more patients with active or recently active tuberculosis requiring a

high degree of health supervision, there is the threat of another 4,000 Navajos with healed reinfection tuberculosis that may intermittently relapse and thus feed the pool of infectious tuberculosis. It would be unrealistic to attempt follow-up examinations or treatments on this large group of patients scattered over the 25,000-square mile Indian country by referring them to the few and widely scattered hospital or dispensary facilities that are equipped to handle their problem. A patient cannot be expected to travel great distances for an essentially prophylactic health measure. What is required here is a carefully planned, community-oriented, field tuberculosis control program which can provide the necessary medical supervision.

Obviously, any field program that is developed for a Navajo community must take into account the very real cultural differences described earlier. In our field health project at Many Farms, community receptivity of modern medicine has been outstanding. We attribute this response to success in overcoming some of the complicated language and cultural barriers. Essentially our approach was this: we made a continous effort to keep the tribal officials, the local leaders, and the Project community fully informed about the nature, purpose, and progress of the medical program; second, we successfully trained bi-lingual Navajos as subprofessional health workers for clinic and field health activities. We have leaned heavily on the ability of these Navajo assistants to interpret our medical program to the community. In turn, the Navajos have been able to convey their views on health matters to our physicians through these same native workers.

Our physicians and nurses working closely with our anthropologist and Navajo colleagues have collected invaluable information about the community. This has included population census, family composition, clan organization, camp locations, mobility of families within and out of the Project area, and socio-economic data about the community. Such information is essential to the organization and development of any effective field health program. Because such data are constantly infused into the medical records and careful observations on the community response to the medical program are available, it is not difficult to keep abreast of the changing pattern of Navajo attitudes, beliefs, and behavior toward modern medicine and their own traditional healing ceremonies.

Navajos have always demonstrated a willingness to modify their traditions if they can be shown that change will lead to improvement. When physicians are also willing to modify some of their "scientific culture," the effective treatment of tuberculosis is assured.

Finally, it should be recognized that further and continuous control not only of tuberculosis, but of all health problems in underdeveloped areas of the world, requires an amalgamation of modern scientific techniques and a sensitivity to and utilization of those elements of indigenous cultures which are meaningful and valuable.

Acknowledgment

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SURGERY IN PULMONARY TUBERCULOSIS[1]

A Review

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In treating a patient with pulmonary tuberculosis, is the goal to rid his lungs of all of the disease? Or is the aim simply to attain a healthy patient who is not a menace to himself or others for the balance of an adequate span of life? For the following remarks to be meaningful, each physician who reads them should be formulating an answer to these questions.

It is widely believed today that chemotherapy and resection together provide essentially all that is needed for the treatment of pulmonary tuberculosis (1). In considerable conflict with this common attitude, the present writers believe not only that several other forms of therapy have merit but, also, that present-day enthusiasm for resection in pulmonary tuberculosis is subsiding to a considerable degree.

As has been true of many other diseases, enthusiasms for different new treatments for tuberculosis have waxed and waned for many years. Not so long ago (1947 to 1949), when the efficacy of chemotherapy was first being broadly recognized, thoracoplasty was just being finally proved to be the most effective treatment for cavitary pulmonary tuberculosis then available *over the long run* (2–11). Long-term proof of the efficacy of any new treatment for tuberculosis was also by then regarded as essential for definitive evaluation.

Following the completion of thoracoplasty before the advent of chemotherapy, it was often six to twelve months before a cavity was lost to view and reversal of infectiousness had occurred; an occasional positive smear or culture months or even years following clinically successful thoracoplasty was not uncommon; parenthetically, such findings did *not* usually herald the onset of overt clinical relapse (12). Furthermore, cavities under thoracoplasty were often not completely healed by either morphologic or bacteriologic criteria when examined at necropsy after death from nontuberculous causes (13, 14). This fact did not cause much concern at the time because some two-thirds of these pre-chemotherapy thoracoplasty patients remained clinically well; in addition, measures to control the active disease often smoldering under a thoracoplasty, i.e., rest, revision thoracoplasty, and resection, were apt to be ineffective, even dangerous.

Soon after the advent of streptomycin and of para-aminosalicylic acid (PAS) treatment in 1946 to 1947, the risks of resecting tuberculous lung were considerably reduced (15–17). Lung resection itself had previously become quite a well-established procedure as a consequence of the introduction of penicillin and the improvements in surgical techniques, pre- and postoperative care, and anesthesia. Hence, when potent tuberculosis chemotherapies were introduced, especially isoniazid, a wave of enthusiasm for resection appeared (3, 18–32).

The pathologic and bacteriologic studies of human lung tissue made available by this enthusiasm have led to much new knowledge of the course of tuberculosis in living patients (14, 33–44). These studies also have generated some new questions which have not yet been answered. For instance, scattered collections of leukocytes are sometimes found in tissue resected after six or more months of theoretically adequate chemotherapy (45). Such findings suggest recent pneumonic spreads of tuberculosis which, in turn, imply that the chemotherapy had been relatively ineffective; this is often in clear contradistinction to the clinical course. A more acceptable explanation of this discrepancy might be that the histologic changes may be a chemical pneumonitis or "tuberculin poisoning" caused by the release of by-products of the rapid destruction of tubercle bacilli (14, 37).

The discovery of intact acid-fast bacilli in various parts of tuberculous lung tissue resected after prolonged chemotherapy has been interpreted to indicate a failure of chemotherapy. On the other hand, because it is seldom possible to

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grow these organisms artificially, they have been regarded either as dead or no longer capable of reproduction (36). Some believe they can be grown artificially under very special circumstances (41). Most observers now believe they are in fact alive, although probably partially damaged; many have believed that, as a consequence, such tissue is best out of the body.

Persons who favor resection for these reasons appear to give little consideration to the possibility that healing might have become complete, or nearly so, if the tissue were examined after additional months of chemotherapy.

By about 1951 to 1952, on the basis of experience with relapse, the resection of "*closed* negative" lesions during original prolonged streptomycin-PAS therapy was beginning to appear unnecessary (16, 23, 46). By 1958, with the passage of time and the use of regimens including isoniazid, it was clear that relapse was low in these cases with a small, statistically insignificantly higher rate of relapse in those whose closed lesions had previously been air-containing than in those whose closed lesions had never been observed to slough (46).

However, among persons who did not have their "open-negative" lesions resected during the initial *streptomycin-PAS* therapy, more than 50 per cent experienced relapse after completing a year or more of initial treatment. By contrast, if the cavity had been removed by resection or collapsed by thoracoplasty, the relapse rate fell to 10 to 15 per cent during the ensuing three to five years (46, 47). The term "open-negative" lesion is here applied to an area which was once roentgenographically a typical tuberculous cavity and which, on prolonged chemotherapy, remains "open" or air-containing, but with complete reversal of infectiousness as determined by repeatedly negative smears and cultures of sputum and gastric washings over a period of three or more months.

Since the introduction of isoniazid, the occurrence of the "open-negative" syndrome has seemed to be on the increase (R.S.M.). The unfavorable experience with streptomycin-PAS in the "open-negative" cases had created a strong indication for surgery in these cases. However, the location and extent of the disease, the age of the patient, or the impaired breathing capacity in practice made resection and often thoracoplasty or plombage thoracoplasty impossible in many of these patients. Others refused surgery, often because they *felt too well* to take the surgical risk.

These patients, who received regimens including isoniazid and achieved the "open-negative" status, have now been followed for two to three years since attaining that status; only about 5 to 10 per cent or less have had relapse by even the strictest criteria (46, 48–50). Some of these open lesions have even gradually become lost to view (48). Secondary infection of these "empty spaces" was anticipated but has actually occurred very infrequently. On the other hand, most of these patients were still receiving isoniazid when last observed. What will happen to them in the long run, especially after all therapy has been discontinued, it is not yet possible to say.

Many "open-negative" lesions have been resected and some have been thoroughly studied. A few—perhaps 10 to 20 per cent of those well studied—have shown true "open healing" (51–55). The term "open *healing*" is used here to describe a tuberculous process which was originally cavitary, which has remained "open" and air-containing by roentgenography and which, upon careful morphologic and bacteriologic examination after resection, reveals nothing to indicate its tuberculous origin, i.e., caseation, epithelioid tubercles, or acid-fast bacilli. "Open healing" was found rarely before chemotherapy and was seen occasionally during the streptomycin-PAS era; only since the advent of isoniazid has it been reported with frequency (R.S.M.). On the other hand, the regimen and duration of chemotherapy, the roentgenographic appearance, and all other *preoperative* findings fail to make the clinical diagnosis of "open healing" entirely reliable.

Enthusiasm for the removal of these "*open-negative*" lesions is still quite widespread today. The argument runs as follows: One cannot be sure on purely clinical grounds whether "open healing" has occurred. One cannot be sure that relapse will not occur. At one time, relapse (in the streptomycin-PAS era) occurred more frequently when "open-negative" lesions were not resected than when they were. Resection can be performed in many of these cases with a minimal risk of morbidity and mortality.

In the opinion of the writers, it is time to reexamine this argument critically. Many of these patients may prove to be *cured* with chemother-

apy alone, especially if the regimen includes *daily* streptomycin for at least three months, and isoniazid (especially in high doses: R.S.M.) and, preferably, PAS too, for no less than eighteen to twenty-four months or more. Morbidity and mortality from surgery in this type of case, as low as they may be, may still be quite unnecessary.

All thoracic surgical operations possess at least some risk of mortality and morbidity. This risk is greater in those who are still infectious at the time of operation, and is still greater in those whose strains of tubercle bacilli have become predominantly drug resistant than in those who are no longer infectious (56, 57). This increased risk of resection in the presence of drug resistance is obviously due, in part, to the fact that drug resistance emerges predominantly in the patient with far advanced chronic cavitary pulmonary tuberculosis with advanced fibrosis, advanced age, poor pulmonary function, and other basically unfavorable features (48). Drug resistance is especially unfavorable when no *effective* new drug coverage is utilized.

It is now well established that resection may be performed safely *early* during original effective chemotherapy (20, 58). In addition, roentgenographic clearing over many months of drug treatment may not reduce the anatomic extent of the resection ultimately required; the "clearing" seen after the first few weeks frequently reflects the *shrinkage* of diseased segments rather than true clearing of disease (48). The fact that the risk of emergence of drug resistance increases with passage of time on drugs is an additional theoretic argument in favor of early resection. These arguments indicate that resection should be performed *early*, i.e., after one to four months of drug treatment, *provided* it is reasonably certain that it will ultimately be indicated.

It can be predicted that some patients will not take their chemotherapy regularly, especially following departure from the hospital. This expectation in individual cases may provide a legitimate indication for resection, even with "closed-negative" lesions. On the other hand, no data are yet available to show whether inadequate chemotherapy *plus* resection is preferable in the long run to inadequate chemotherapy alone.

Another phenomenon of modern chemotherapy is the apparent failure of large cavities to respond to collapse. In the mid-forties and earlier, collapse was capable of closing cavities and reversing infectiousness, and patients who had had such therapy might remain well for years. Pneumothorax, for instance, was often successful in closing small, thin-walled cavities (59). Phrenic nerve interruption was occasionally effective in highly selected cases. Pneumoperitoneum was moderately effective in selected cases and, incidentally, quite safe (37). Thoracoplasty was by all odds the most effective collapse method, especially when it is realized that it was usually not applied until after failure of one or more of the temporary forms of collapse (59).

Control of cavitary tuberculosis was largely accomplished in pre-chemotherapy days by means of occlusion of the bronchocavitary junction, with consequent inspissation of the cavity contents (14). The blood- and oxygen-free medium of the inspissated cavity tended to cause a gradual death of its sequestered tubercle bacilli (14). Before chemotherapy, effective collapse probably closed the bronchocavitary junction by kinking or compressing an inflamed or damaged bronchus. In combination with mildly effective chemotherapy, collapse seemed also to work out well (60–71), apparently still by closure of the bronchocavitary junction and inspissation of the cavity contents.

Today the collapse picture is apparently different. Collapse appears to add little to original, uninterrupted, highly effective chemotherapy (48). In patients receiving such treatment, the bronchial ulcerations are rapidly, i.e., sometimes within two weeks, healed and re-epithelized (34). This rapid healing makes it unlikely that the bronchocavitary junction will cease functioning as a result of kinking or growing together. When inspissation occurs under these circumstances, it is presumably because the cavity contents do not become sufficiently fluid to flow out into a widely patent bronchus. The contents of these cavities therefore usually empty into draining bronchi and are expectorated, swallowed, or deposited in other parts of the lungs. The cavity then may heal and disappear. If large enough, and particularly if the surrounding lung is inelastic enough, an empty space will remain and will often even enlarge. This sequence of events is presumed to account for the increased frequency of the "open-negative" syndrome in the last three or four years (47, 49, 52–55, 72–76).

Parenthetically, these observations do not mean that collapse does not have any place in

the treatment of tuberculosis today. Thoracoplasty and extraperiosteal plombage thoracoplasty may be effective in chemotherapy failures; and especially advisable in those patients with drug resistance, in those who do not lend themselves anatomically to resectional surgery, and in those with grossly impaired pulmonary function. In short, healing by closure of the bronchocavitary junction and by inspissation of the cavity contents may still be anticipated in chemotherapy failures.

Plombage fell into disrepute because of complications resulting from intrapleural and extrapleural placement of various plombage materials. The temporary or permanent *extraperiosteal* plombage, however, is not only an effective collapse measure, but complications occur quite infrequently today (11, 77–86).

On principle, it is preferable to remove the foreign material at a second operation four to eight months following the first. At this time a resection may also be performed if the objective of the plombage has not been achieved, and the risk is then acceptable (86). In older patients, a single-stage permanent plombage may be preferred to the two-stage temporary plombage.

Both pneumothorax and pneumoperitoneum might also logically be revived in treating chemotherapy failures, except that both procedures have been shown to be relatively unsuccessful in treating the chronic fibroid form of the disease in which most of these chemotherapy failures present.

Despite the absence of clinical evidence of active disease, the possibility that a patient may still be infectious to persons in close contact with him should always be considered. Experience has shown that open cavitary disease is the major source of contagion. The tuberculosis morbidity of contacts of persons with noncavitary pulmonary tuberculosis was no higher than the rate observed in the general population (87). Furthermore, a rather remarkable phenomenon has occurred with the use of isoniazid. After isoniazid therapy, tubercle bacilli often appear to lose some of their pathogenicity and infectivity for humans (88–90). This observation was first made in guinea pigs (91), and later in other experimental animals. Tubercle bacilli which are highly resistant to isoniazid and which have also lost their catalase activity, probably also have a greatly reduced capacity for transmitting tuberculosis to humans. In support of this opinion, well-documented cases of primary highly isoniazid-resistant and catalase-negative infections have been only rarely reported in the six years since the introduction of the drug (92–95). Furthermore, while new areas of disease may occur in persons who excrete a pure population of these mutants, these new shadows usually clear quite rapidly (88). Tuberculous complications of surgery in such persons may therefore be presumed to occur less frequently than in those with infections less resistant to isoniazid, catalase-positive infections, and in those with isoniazid-susceptible infections without effective chemotherapy. It is also possible—even probable—that many of the chronically ill persons who have received a great deal of isoniazid and who continue to receive the drug (to prevent possible back mutation to isoniazid susceptibility) (96) may be *relatively* noncontagious.

The paucity of reports of primary isoniazid-resistant infections is not matched by the numerous reports of primary streptomycin- or PAS-resistant infections, which have represented 2 to 5 per cent of newly diagnosed disease from various parts of the world for the past ten years (94, 97–102).

"Tuberculous bronchiectasis" is regarded by some to be an indication for resectional surgery (103–105). Pathologists have repeatedly emphasized that true bronchiectasis rarely, if ever, occurs as a complication of tuberculosis (14, 33, 34, 37). True bronchiectasis is characterized not only by dilatation, but also by ulceration and inflammatory infiltration of the bronchial walls and by loss of integrity of the muscularis and even thromboses of the adjacent pulmonary vessels. These characteristic findings are rarely found in the cylindrically dilated, foreshortened and crowded bronchi in shrunken, healing tuberculous segments or lobes (14, 33, 34), i.e., so-called "tuberculous bronchiectasis." Furthermore, these bronchial changes, commonly observed in association with healing tuberculosis, are not commonly associated with later breakdown or relapse when adequate chemotherapy has been used (48).

That modern chemotherapy regimens are not all equally effective has been eminently demonstrated by numerous studies, both controlled and otherwise (23, 24, 29, 46, 106–115). Daily streptomycin plus isoniazid plus PAS may be regarded as the regimen of choice in "panic" situations such as tuberculous meningitis and very far

advanced pulmonary tuberculosis. This regimen is obviously not necessary in many cases of tuberculosis; however, one cannot always be sure at the outset what is the immune balance in the individual; furthermore, very prompt reversal of infectiousness appears to reduce the risk of the emergence of drug resistance (12).

A few persons continue to excrete a population of tubercle bacilli predominantly susceptible to a drug or drugs after prolonged use of those drugs. This may be due to an effective combination of two or more agents without interruption. It may also represent evidence of failure to take the drug, failure to absorb it into the blood, or failure to achieve adequate concentrations of the drug in diseased tissues, possibly because of excessive inactivation (12).

* * *

To sum up: The writers are convinced that, in tuberculosis therapy, the goal should be a healthy patient who is also not able, or is unlikely, to infect those in his environment. Attempts should be made to eradicate tuberculosis from the lungs, but *not surgically*, since it is obviously impossible to remove every tuberculous focus in the lungs. The persistent opinion of some observers that, in persons who are no longer infectious and who are receiving good chemotherapy resection is indicated in the larger closed tuberculous lesions, especially those which were previously cavitary, should be re-examined. The present widely-held view that "open" lesions should be surgically resected or collapsed in persons who are otherwise well and noninfectious for many months during effective chemotherapy should also be re-examined. The generally accepted policy to postpone surgery in most cases for six to eight months, or until maximal improvement has been attained from chemotherapy, should likewise be scrutinized. Finally, the greater part of our attention should be concentrated on the *treatment and prevention* of chemotherapy failures, i.e., those persons who remain infectious and, particularly, those whose strains of tubercle bacilli have become predominantly drug resistant.

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SOME FRACTIONS OF TUBERCLE BACILLUS WAX AND THEIR IMMUNOGENICITY FOR MICE[1, 2]

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Introduction

Purified tubercle bacillus wax (1), dissolved in *n*-hexadecane and suspended in water, has shown effective immunizing powers against mouse tuberculosis. This wax contains many highly extracted and almost completely nonacid-fast mycobacteria. High speed centrifugation of wax in an *n*-octane solution concentrated most of these organisms into an immunogenic high-speed wax sediment, while the supernate failed to enhance resistance (2). The present report concerns further studies on fractions of Anderson's purified tubercle bacillus wax, hereafter designated as "Wax."

Materials and Methods

Wax in concentrations up to 25 mg. per ml. formed lightly turbid solutions at room temperature in *n*-heptane and *n*-octane. Redistilled *n*-heptane was mainly used, as it is lighter (specific gravity 0.684 at 20°C.) than *n*-octane and evaporates faster. High-speed centrifugation (35,000 r.p.m., one to three hours) broke such Wax solutions sharply into (I) high-speed sediment which contained practically all of the extracted bacteria; and (II) high-speed supernate. The high-speed sediment, when dried, was amber in color and brittle in consistency. The high-speed supernate remained in an almost clear solution and dried to resemble a light-colored, stiff grease.

Low-speed centrifugation of Wax or high-speed sediment solutions, which were run two or more times at approximately 2,000 r.p.m. for ten minutes, threw down small amounts of (III) low-speed sediment which (presumably) contained most of the bacteria plus some other waxy material. The corresponding supernates constituted either (IV) Wax minus low-speed-sediment or (V) high- minus low-speed sediment.

Heptane solutions of Wax minus low-speed sediment and high- minus low-speed sediment were easily filtered through 300 mμ (PH) and/or 450 mμ (HA) Millipore filters, but only nonmeasurable traces of these fractions could be drawn through 100 mμ (VC) filters. It was possible, however, to pass high-speed supernate through (VC) filters in small but measurable amounts.

All fractions were dried, some in a vacuum desiccator but most under flowing nitrogen.

The main work here reported involves six experiments on the immunizing properties of Wax or its fractions against tuberculosis in mice. Most techniques have been described (2–4). There were 30 mouse groups and each group contained separate batches of approximately 17 males and 17 females which were four to seven weeks old when first vaccinated. Each experiment included a group of untreated controls, and no other controls were used (2).

The vaccinated mice received two subcutaneous injections of Wax or a fraction at 14 ± 1-day intervals. All vaccines were dissolved (or suspended) in purified *n*-hexadecane[3] and emulsified before use by shaking in water. The individual vaccine doses were 0.2 mg.[4] in the first four and 0.1 mg. in the last two experiments. These doses were contained in 1 ml. of 1 per cent hexadecane for the first test and in 0.5 ml. of 2 per cent hexadecane for all other experiments.

Intravenous challenge with H37Rv followed the second vaccine dose by 14 ±1 days. All challenge volumes were 0.25 ml. H37Rv was grown approximately two weeks on Löwenstein-Jensen slants and grossly dry cultures were selected. A bacterial mass was weighed and ground in distilled water only for the first experiment. Grinding was then begun in full-strength mouse serum, and water was gradually added to make a final 1 per cent serum concentration. Challenge suspensions contained smaller gross clumps and retained their original uniformity longer when ground with serum than when water comprised the sole suspending medium. The challenge dose was 1 mg. of H37Rv in the first experiment, 0.75 mg. for the next four, and 0.375 mg. in the last test.

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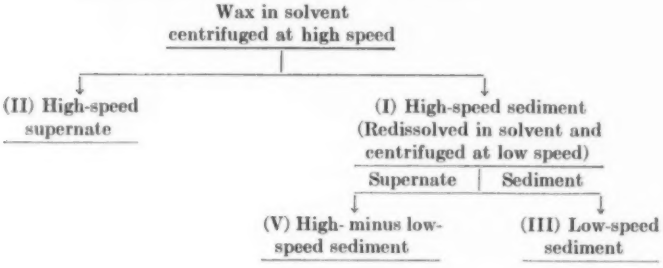
[2] Supported in large part by research grants (E-661 and E-2042) from the Institute of Allergy and Infectious Diseases of the National Institutes of Health, U. S. Public Health Service.

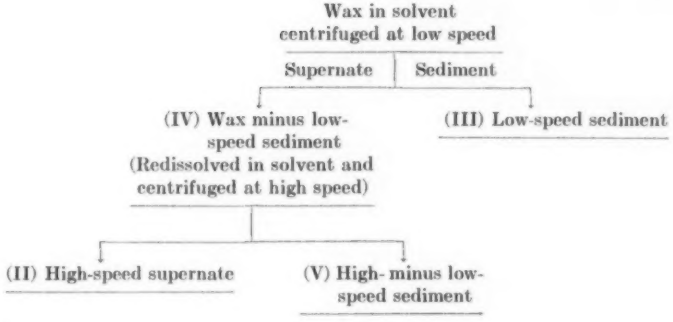
[3] *n*-Hexadecane and other hydrocarbons were kindly supplied by Mr. Bingham J. Humphrey, Humphrey-Wilkinson, Inc., DeVine Street, North Haven, Connecticut.

[4] Mice received two 0.04-mg. doses of low-speed sediment in one test, due to shortage of material.

Fractionation Diagrams (omitting filtration)



or


Wax in solvent
centrifuged at low speed
Supernate
Sediment
(IV) Wax minus low-speed sediment (Redissolved in solvent and centrifuged at high speed)
(III) Low-speed sediment
(II) High-speed supernate
(V) High- minus low-speed sediment

Experiments were held until no more deaths were imminent, approximately four to six weeks after challenge, and all mice (except a few too much destroyed by cannibalism) were examined at autopsy after death or when the tests were terminated.

Mouse groups were compared for differences between mean days to death and also for differences between numbers surviving at the end of the tests (survival percentages) (2, 3). Logarithmic transformations of time were used as before (2–4); hence, the mean days to death are geometric means or antilogs of mean logarithms.

Comparison was first made of sex differences within each treated and control group. Sex differences in resistance have been noted (3), but these differences were minimal following subcutaneous vaccination (2), and the present findings confirm previous observations. Hence, and also because male and female batches in each group were essentially equal in size, the two sexes of every group were added for final intergroup comparisons. All experiments available for comparing any treatment with its untreated controls, i.e., 5 tests including high-speed supernate vs. corresponding controls, or with any other treatment, were pooled.

Results

The power to immunize mice and some general characteristics[5] of Wax and its fractions (omitting filtered fractions) are outlined in table 1. The remainder of this section concerns tests for immunogenicity of Wax and Wax fractions.

Mice that were vaccinated with Wax or its fractions, omitting filtered vaccines, are compared with their controls in table 2.

The percentage increases of treated over corresponding control mice in table 2 are shown below:

Mean Days to Death	*Experimental Groups*	*Number Surviving*
0.0	(II) High-speed supernate	−2
13.5	(V) High- minus low-speed sediment	11
13.1	(IV) Wax minus low-speed sediment	7
17.6	Wax	13
22.4	(III) Low-speed sediment	31
25.6	(I) High-speed sediment	19

[5] Total nitrogen determinations were run by the Elek Micro Analysis Laboratories, 4763 West Adams Boulevard, Los Angeles 16, California.

TABLE 1

IMMUNIZING POWER FOR MICE AND GENERAL CHARACTERISTICS OF WAX

Wax or Fractions	Power to Immunize Mice	Solubility in Normal Hydrocarbons at Room Temperature (unless slight heating is specified)		Melting Points	Total Nitrogen	Per Cent of Wax Weight
		Heptane, Octane	Hexadecane			
Wax	+	Lightly turbid	Almost clear with slight heating	Melts with decomposition 51°–180°C.*	Not done	100
(IV) Wax minus low-speed sediment	+	Lightly turbid	Almost clear with slight heating	Melts with decomposition 67°–164°C.; after one purification, 203°–206°C.†	Not done	Not done
(I) High-speed sediment	+	Lightly turbid	Almost clear	Not done	Not done	Not done
(V) High- minus low-speed sediment	+	Lightly turbid	Almost clear	Melts with decomposition 155°–205°C.	1.27%	68 to 69
(III) Low-speed sediment	+	Turbid suspension	Turbid suspension	Not done	Not done	1 or less
(II) High-speed supernate	−	Almost clear	Almost clear with slight heating	48°–49°C.	0.99%	30 to 31

* Further purification by precipitation from toluene with methanol produced two fractions: An oily layer, separated and purified by precipitation with methanol, melted at 203°–207°C. Further addition of methanol to original mother liquor produced a white precipitate melting at 46°–48°C.

† Purified by precipitation from chloroform with ethanol.

It is obvious that the resistance of mice was enhanced significantly by vaccination with Wax and all fractions save high-speed supernate. This is shown by an increase in both mean days to death and numbers surviving until the end of the experiments for the vaccinated mice relative to their controls.

High-speed supernate failed to show any immunizing potency. From the observed standard error of the difference in means of mice that died, there is a calculated 95 per cent confidence that this fraction cannot increase length of life by more than 3 per cent over that of the controls. Since this maximal degree of immunizing potency is negligible, these data exclude any appreciable immunogenicity of the high-speed supernate.

When vaccinated groups (omitting high-speed supernate) were compared with each other, only one significant difference appeared. In four pooled experiments, 27 of 132 mice vaccinated with high- minus low-speed sediment survived in comparison with 16 survivors of 136 vaccinated with Wax minus low-speed sediment ($p < 0.05$). The difference in mean days to death (18.8 vs. 18.1) pointed in the same direction, but was not significant.

Wax contains many extracted mycobacteria which are almost entirely nonacid-fast (2); but when Wax is dissolved in a light solvent and centrifuged two or more times, these organisms become increasingly atypical, disintegrated, and hard to identify. It is therefore difficult to be sure that low-speed centrifugation has removed most of these bacteria or their fragments from Wax minus low-speed sediment or from high- minus low-speed sediment. Hence, these fractions were passed by suction through 300 mμ Millipore filters and a duplicate solution of Wax minus low-speed sediment was run through a 450 mμ

TABLE 2

SUMMARY OF WAX FRACTIONATION EXPERIMENTS OMITTING FILTRATION

Males and Females Combined

Number of Experiments	Experimental Groups	Total Mice	Mice that:		Significant p Values (0.05 or Less) between Experimental and Control Groups by	
			Died (Mean Days to Death)	Survived (Numbers Surviving)	Mean Days to Death	Numbers Surviving
5	(II) High-speed supernate	163	15.7	7	—	—
	Controls	161	15.7	10		
5	(V) High- minus low-speed sediment	160	17.7	27	<0.001	0.001
	Controls	162	15.6	9		
4	(IV) Wax minus low-speed sediment	136	18.1	16	<0.001	<0.05
	Controls	134	16.0	6		
3	Wax	91	17.4	17	<0.001	<0.01
	Controls	88	14.8	5		
2	(III) Low-speed sediment	55	18.6	24	<0.001	<0.001
	Controls	63	15.2	8		
1	(I) High-speed sediment	37	19.6	8	<0.001	<0.05
	Controls	33	15.6	1		

TABLE 3

WAX FILTRATION EXPERIMENTS

Males and Females Combined

Experimental Groups	Total Mice	Mice that:		Significant p Values (0.05 or Less) between Experimental and Control Groups by	
		Died (Mean Days to Death)	Survived (Numbers Surviving)	Mean Days to Death	Numbers Surviving
Unfiltered (IV) Wax minus low-speed sediment	72	17.6	8	0.02	<0.01
Filtered (IV) Wax minus low-speed sediment	72	19.3	12	<0.001	<0.001
Unfiltered (V) High- minus low-speed sediment	70	19.1	8	<0.001	<0.01
Filtered (V) High- minus low-speed sediment	72	18.7	9	<0.001	<0.01
Controls	72	16.1	0		

filter. The immune levels evoked by mouse vaccination with both of these filtered[6] fractions and their unfiltered aliquots were assayed in two experiments, the findings of which are pooled in table 3.

The percentage increases of treated over corresponding control mice in table 3 are shown

[6] Mice received Wax minus low-speed sediment which had passed through a 450 mμ filter in one experiment, and through 300 mμ pores in the second test. High- minus low-speed sediment was filtered through 300 mμ pores for both experiments.

below:

Mean Days to Death	Experimental Groups	Number Surviving
9	Unfiltered Wax minus low-speed sediment	11
20	Filtered Wax minus low-speed sediment	17
19	Unfiltered high- minus low-speed sediment	11
16	Filtered high- minus low-speed sediment	12

Mice vaccinated with both filtered and unfiltered portions of Wax minus low-speed sediment and high- minus low-speed sediment show significantly increased resistance levels relative to their untreated controls. No significant differences in resistance appear between any of the vaccinated groups.

Discussion

At the onset of this work the writers believed that the immunizing ability of their supply of Anderson's purified tubercle bacillus wax lay mainly, if not entirely, in the extracted mycobacteria or bacterial fragments which it contained. However, the processes of low-speed centrifugation and filtration to which Wax and high-speed sediment were subjected in producing filtered Wax minus low-speed sediment and high- minus low-speed sediment should have removed a high proportion of these bacterial formed elements. In spite of these procedures, the filtered fractions retained their definite power to immunize mice against experimental tuberculosis.

It now seems logical to believe that all Wax fractions, save high-speed supernate, comprising approximately 30 per cent of the Wax, bear immunizing components that are not due to contamination with mycobacteria. This belief is strengthened by Crowle's report (5) that a number of filters, including 450 mμ (HA) Millipore filters, can remove live tubercle bacilli from suspension in light hydrocarbons.

The writers' original Wax supply was probably prepared before 1927, as Anderson filtered tubercle bacillus extracts through paper in his early work reported in 1927, but thereafter employed Chamberland filters (6, 7). This wax presumably picked up moisture and underwent some chemical decomposition during its long storage period (8), for its present melting range extends considerably below that originally reported.[7] Despite any chemical decomposition, however, the present work shows that Wax and all but one of its fractions can still build up measurable levels of increased resistance in mice when injected with *n*-hexadecane. The immunologic stability of Wax must be high.

Anderson's purified tubercle bacillus wax is the reprecipitated portion of a thorough chloroform extraction of tubercle bacilli after removal of a prior ethanol-ether extract. It has been further split by precipitation with hot acetone into Wax C, a low melting fraction, and Wax D composed mainly of high melting lipopolysaccharides (9, 10). The present writers' high-speed supernate appears to be chiefly Wax C while their immunogenic high-speed and high- minus low-speed sediments are thought to be principally Wax D. These immunogenic sediments cannot contain much protein, for they were derived from a chloroform extract with an expectedly low protein content, and a total nitrogen determination was also low. However, traces of protein can be adsorbed on or conjugated with lipopolysaccharides, and lipopolysaccharides can be adsorbed on small, filter-passing fragments of extracted tubercle bacilli. Such protein traces might activate lipopolysaccharides into full, immunizing antigens.

The writers' supply of Anderson's Wax contained a wide range of particles of different sizes. The largest were microscopically visible structures and other macromolecules which fell into the low-speed sediment. A smaller range of particles was sedimented out of a lightly turbid colloidal solution by high-speed centrifugation, while an almost clear solution of still smaller particles remained in the high-speed supernate. This latter fraction bore no immunizing power and might well be removed from vaccines made of Wax fractions.

The writers' Wax supply has run low and little more of Anderson's purified tubercle bacillus wax is apparently available. It is therefore impossible to prepare another lot of low-speed sediment from Anderson's wax in an amount sufficient for further testing. This small fraction, comprising the largest particles in the Wax,

[7] Dr. Esmond R. Long kindly supplied 5 gm. of purified tubercle bacillus wax from the Henry Phipps Institute, Philadelphia, Pennsylvania, in 1955. This wax was an original preparation by Dr. R. J. Anderson, but there is no definite record of when this lot of wax was prepared.

may be a superior immunizing agent, but this was not proved.

Many investigators have prepared and tested experimental, nonviable vaccines against tuberculosis and only a small part of this work can be mentioned here. As recently emphasized by Weiss (11), such vaccines can confer on animals a range of immunity comparable to that produced by live BCG or the vole bacillus. Dubos, Weiss, and Schaedler (11–16) extracted an immunizing fraction with hot absolute methanol from phenol-killed and acetone-washed tubercle bacilli. This fraction produced active and long-lasting immunity in mice and some immunity without tuberculin sensitivity in guinea pigs. It was less toxic but also less immunogenic than the cells from which it stemmed. Its immunizing action was intensified by several adjuvants.

Killed tubercle bacilli apparently contain not only immunizing but also immunity-inhibiting or toxic components (10, 17). Also, dependent on conditions, vaccination with such organisms may either alleviate or accelerate the course of other infections (14, 15)—an example of many surprising and analogous phenomena. Smith and Kubica (18) made ethanol extracts of mycobacteria followed by ethanol-ether, chloroform or methanol-chloroform extractions, and the latter extract was sometimes subfractionated by precipitation at low temperatures. Final separations were made by column chromatography. Their fractions were tested in guinea pigs either alone or with defatted bacilli; and certain fractions or combinations, as well as bacilli defatted by neutral organic solvents, produced promising immune levels.

A different type of work involves the physical concentration of certain parts of tubercle bacilli. Here may be mentioned the highly sensitizing cell wall fraction of Ribi and associates (19) and the immunogenic intracellular granules of Youmans and co-workers (20).

Extractive fractionation of tubercle bacilli and tests on the immunizing values of these fractions, along with the use of adjuvants to improve their action, particularly interest the present writers, and their work in progress points along these lines.

Summary

Anderson's purified tubercle bacillus wax was dissolved in light hydrocarbons and separated into five fractions by differential centrifugation followed by drying.

Vaccination with aqueous *n*-hexadecane suspensions of Wax and four fractions significantly immunized mice against tuberculosis. The fifth fraction, approximating 30 per cent of the Wax, proved immunologically inert.

Wax contained extracted mycobacteria and bacterial fragments. Samples of two immunogenic fractions were passed through Millipore filters to remove all bacteria possible. This filtration did not lower the immunizing powers of these fractions.

Sumario

Algunas Fracciones de La Cera de los Bacilos Tuberculosos y su Inmunigenicidad para los Ratones

Se disolvió la cera purificada del bacilo tuberculoso de Anderson en hidrocarburos livianos, separándola en cinco fracciones por la centrifugación diferencial seguida de la desecación.

La vacunación con suspensiones *n*-hexadecánicas acuosas de cera y cuatro fracciones inmunizó significativamente a los ratones contra la tuberculosis. La quinta fracción, que formaba próximamente 30 por ciento de la cera, resultó ser inerte inmunológicamente.

La cera contenía micobacterias extraídas y fragmentos bacterianos. Muestras de dos fracciones inmunógenas fueron pasadas a través de filtros miliporosos para eliminar todas las bacterias posibles. Esta filtración no rebajó las potencias inmunizantes de estas fracciones.

Resume

De quelques fractions de cire de bacille tuberculeux et de leur immunogénicité pour la souris

La cire de bacilles tuberculeux purifiée de Anderson a été dissoute dans des hydrocarbures légers et séparée en cinq fractions par centrifugation différentielle suivie de séchage.

La vaccination avec des suspensions aqueuses de *n*-hexadecane de la cire et de quatre des fractions a immunisé les souris contre la tuberculose de façon significative. La cinquième fraction, approximativement 30% de la cire, s'est montrée inerte du point de vue immunologique.

La cire contenait des mycobactéries extraites et des fragments de bactéries. Des spécimens des deux fractions immunogéniques ont été filtrés à travers des filtres millipores pour enlever toutes les bactéries qui pouvaient s'y trouver. Cette

filtration n'abaissait pas le pouvoir immunisant de ces fractions.

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Grateful acknowledgment is made to Dr. John W. Mehl, Department of Biochemistry and Nutrition, School of Medicine, University of Southern California, for his advice and his permission to use certain facilities, and particularly an ultracentrifuge in his department.

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NUTRITIONAL STUDIES OF ISONIAZID IN RATS DEFICIENT IN VITAMIN E[1]

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INTRODUCTION

Two of the theories which have been advanced to explain the antituberculous activity of isoniazid appear—at least at first sight—to be incongruous. Thus, some authorities (1–3) have suggested that isoniazid harms those types of tubercle bacilli which are susceptible to this agent by the intracellular accumulation of hydrogen peroxide, possibly through the faulty formation of catalase or peroxidase. This view has recently been challenged by Holmes and Rubbo (4). The other theory is due to Bergel (5–7), who considers that isoniazid and certain other drugs used in the treatment of tuberculosis or leprosy owe their activity to antioxidant activity. This action could be demonstrated, in Bergel's experience, by the ability of the drug to replace vitamin E, itself a potent antioxidant, in the diet of rats. Thus, he found that isoniazid prevented the brown discoloration of the uterus which regularly develops in deficiency of vitamin E (8). Brown coloration of the body fat, which occurs in association with peroxide formation when deficiency of vitamin E is combined with the feeding of substantial amounts of cod-liver oil (9), in Bergel's tests, could also be prevented by isoniazid. In male rats isoniazid was effective against degeneration of the testes.

Bergel concluded that the autoxidation of fats may be an essential feature of mycobacterial infections, and that isoniazid and certain other drugs act as antioxidants. It is already well known, of course, that various artificial redox substances, typified by methylene blue, can act as partial substitutes for vitamin E (10, 11). According to Granados, Aaes-Jorgensen, and Dam (12), sulfaguanidine protects against the brown coloration of the rat's body fat induced by cod-liver oil, but not against depigmentation of the incisor teeth, another effect of vitamin E deficiency (13).

It seemed advisable, therefore, to obtain further evidence of the action of isoniazid in rats submitted to experimental vitamin E deficiency. If the drug promotes the formation of hydrogen peroxide, an aggravation of the symptoms of deficiency might reasonably be expected. If it acts as an antioxidant, prevention or amelioration of the symptoms of deficiency should result.

The claims of Bergel, it must be emphasized, have been based on experiments in which the basal diet contained cod-liver oil. The use of this oil introduces a complication, since it is now clear that it is both a source and an antagonist of vitamin E (14*a*). Any effect of isoniazid shown in conjunction with this diet might well result from synergistic action between the drug and the vitamin naturally present in the oil, rather than from vitamin potency exerted by the drug itself. In the experiments of the present writers, therefore, the drug was tested not only on rats given a basic diet containing cod-liver oil, but also on others given a diet containing lard. Chemical tests by R. J. Ward of this laboratory have shown that this fat, as supplied to the laboratory, is virtually devoid of tocopherols. Also included in the work were tests on nicotinamide, chosen as a substance resembling isoniazid in structure, but known to have only very little antituberculous activity in laboratory animals (14*b*).

MATERIAL AND METHODS

In several experiments isoniazid was included in the diet of piebald rats of both sexes from the time of weaning for periods up to five months. Other rats received diets without the drug, with the drug replaced by nicotinamide or by adequate supplements of DL-α-tocopheryl acetate, 1 or 2 mg. per rat weekly. In the experiment on nicotinamide, albino rats were used in addition to piebalds.

A "lard diet" contained casein, 25 per cent; sucrose, 50 per cent; lard, 10 per cent; dried yeast, 10 per cent; and minerals, 5 per cent. Adequate weekly supplements of vitamins A, D, and K were allowed.

A "cod-liver oil diet" differed; it contained 10 per cent of good quality cod-liver oil in place of the lard, which made the weekly supplements of vitamins A and D unnecessary.

[1] From Dunn Nutritional Laboratory, University of Cambridge; and Medical Research Council, Cambridge, England.

The diets were made up fresh each week and were supplied *ad libitum*. The animals were weighed and inspected at frequent intervals.

Hemolysis tests for vitamin E status were made on the animals during life by a modification of the method of Gyorgy and Rose (15). Washed erythrocytes, obtained from the tip of the tail, were treated with dialuric acid and were incubated for a standard time. Intact erythrocytes were then removed by centrifugation, and the percentage hemolysis was estimated by comparing the red color of the solution with another solution in which cells from the same rat had been completely hemolyzed with distilled water.

At autopsy the upper incisor teeth were inspected, and any deficiency in the normal brown pigment was noted. Moore and Mitchell (16) have reported that the teeth of piebald rats are less sensitive to depigmentation, at least in avitaminosis E, than those of albinos. In the present work, therefore, comparisons were made only between animals of the same species.

Brown coloration of the uterus was marked according to the following arbitrary scale:

0—Normal uterus; no brown or yellow color in visible light; fluorescence under ultraviolet irradiation, the normal dull blue or mauve.

1—Barely perceptible brown color in visible light. Confirmation by yellow-brown fluorescence under ultraviolet irradiation (17).

2—Definite pale yellow-brown color in ordinary light.

3—Light brown.

4—Chestnut or dark brown. In doubtful cases, markings of ½, 1½, 2½, or 3½ were given.

Testes were weighed, which usually gave an adequate index of degeneration. In doubtful cases, paraffin sections, stained with hematoxylin and eosin, were prepared.

Body fat was inspected in the omental, subcutaneous, and peri-testicular or peri-uterine regions. The same system of marking was employed as for the uterus, except that the presence of the strongly fluorescent vitamin A prevented satisfactory examinations under ultraviolet irradiation.

Kidney sections were made in some experiments in order to study the rate of autolytic histolysis, which is greatly increased by the absence of vitamin E from the diet and also by the presence of certain unsaturated fats (18–21). The kidneys were removed and fixed in formol saline after the animals had been kept at room temperature for three hours after death. Paraffin sections were prepared and stained with hematoxylin and eosin. The degree of autolysis, as indicated by the extent of the areas in which the cells lining the proximal convoluted tubules had disintegrated, was assessed by comparison with standard sections (22).

Results

Isoniazid Dosage with the Diet Containing Lard

In three series of experiments, isoniazid was included in the diets of piebald rats at concentrations of 0.15 or 0.2 per cent for periods of 100 to 168 days. Most of the rats remained in normal health. Weight increases varied between individual animals. The ranges of increase for both sexes, however, were similar for undosed animals and for those dosed with isoniazid, with higher ranges for those dosed with tocopherol (table 1). Doses of 2 per cent of isoniazid were also tried, but proved highly toxic.

Hemolysis tests: In tests on 15 rats dosed with isoniazid, hemolysis percentages of 72 to 100 were found (average, 87.7). For tests on 9 undosed rats, percentages were 79 to 93 (average, 85.6), and for 6 rats dosed with tocopherol, 0 to 15 (average, 3.5). The drug had therefore no vitamin E potency according to this criterion.

Testes and uterus: The effects of the various treatments on the weight of the testes or the degree of brown color of the uterus are shown in table 1. It may be seen that the average testes weight of 1.56 gm. for the animals given isoniazid was higher than for undosed animals (1.27 gm.) but much lower than for the animals dosed with tocopherol (2.81 gm.). The difference between the isoniazid-dosed and the undosed animals depended on three rats among the former, as against only one in the latter, having testes approaching normal size; it therefore seems insignificant.

In undosed females the range of marks for brown coloration of the uterus was 3 to 4 (average 3.3). For females given tocopherol the mark was invariably 0. For females dosed with isoniazid, the range was 1.5 to 2.5 (average 1.9). The difference between the undosed animals and those given isoniazid would certainly be significant, on a mathematical basis, but it must be remembered that marking was on an arbitrary basis. Photographs of uteri, typical of the different groups, are shown in figure 1.

Teeth: In 19 undosed rats, some degree of mottling of the normal brown pigment was seen on the incisors of 12 animals. In 18 rats given isoniazid, mottling was observed in 2, and in 15 rats dosed with α-tocopherol only a single white tooth unexpectedly occurred. Judged on this criterion, therefore, isoniazid seemed effective in replacing vitamin E.

TABLE 1

ISONIAZID DOSAGE. OBSERVATIONS ON RATS FED UPON A DIET CONTAINING LARD

Undosed Rats				Rats Dosed with α-Tocopherol				Rats Dosed with Isoniazid				
Number	Days on Diet	Body Weight Gain	Weight of Testes (gm.) or Uterus Marking	Number	Days on Diet	Body Weight Gain	Weight of Testes (gm.) or Uterus Marking	Number	Per Cent of Drug	Days on Diet	Body Weight Gain	Weight of Testes (gm.) or Uterus Marking
♂		gm.		♂		gm.		♂			gm.	
1	128	234	1.36	20	128	264	2.46	35	0.15	129	203	0.94
2	128	173	0.89	21	128	254	2.63	36	0.15	129	211	0.92
3	128	260	0.99									
4	144	221	1.24	22	145	354	3.15	37	0.20	141	193	1.06
5	144	229	2.51					38	0.20	141	202	2.06
6	165	198	1.16	23	165	314	2.71	39	0.20	165	213	2.93
7	165	187	1.06	24	165	341	2.99	40	0.20	165	204	1.04
8	165	276	1.01	25	165	356	3.00	41	0.20	165	245	0.98
9	165	266	1.17	26	165	275	2.60	42	0.20	165	230	2.88
10	165	290	1.28	27	165	335	2.92	43	0.20	165	218	1.20
Average		233	1.27			312	2.81				213	1.56
♀				♀				♀				
11	99	120	3.0					44	0.15	100	118	2.0
12	106	105	3.0	28	111	170	0	45	0.15	100	146	1.5
13	106	129	3.0	29	111	175	0					
14	141	111	3.5	30	145	157	0	46	0.2	141	80	1.5
15	141	110	3.0					47	0.2	141	82	1.5
16	158	147	4.0									
17	168	147	3.5	31	168	189	0	48	0.2	168	167	2.0
18	168	192	3.5	32	168	180	0	49	0.2	168	138	2.5
19	168	111	3.5	33	168	210	0	50	0.2	168	168	2.0
				34	168	209	0	51	0.2	168	118	2.0
								52	0.2	168	123	2.0
Average		130	3.3			184	0				127	1.9

Renal histolysis: Kidneys were taken from rats which had received their diets for 165 to 168 days. Histologic examination indicated clearly that dosing with isoniazid prevented the rapid post-mortem autolysis which is typical of avitaminosis E. Thus, in 9 undosed rats, histolysis was invariably observed, with markings of 1–2½ (average 1.9). (Marking for maximal histolysis = 3.) Slight histolysis was observed in only 2 of 10 rats dosed with isoniazid, as 0.2 of their diet, with marks of 1 and 1½, and with an average of only 0.25 for the group. Sections typical of each group are shown in figure 2.

Nicotinamide Dosage with the Diet Containing Lard

For 121 to 129 days, piebald or albino rats were given the diet containing lard either without supplements, with doses of tocopherol, or with the addition to the diet of 0.5, 1.0, or 2.0 per cent of nicotinamide. Weight increases were usually slower in the rats given nicotinamide than in the control groups, even when tocopherol was not allowed.

Hemolysis: In 3 rats given 2 per cent of nicotinamide, the percentages of hemolysis were 86, 94, and 94; and in 3 others given 1 per cent of nicotinamide, the percentages were 80, 94, and 100. For an undosed animal, the percentage was 82; and for an animal dosed with tocopherol, 0.4. Nicotinamide therefore had no vitamin E potency according to this criterion.

Testes and uterus: There was no evidence that the effects on these organs of the different levels of nicotinamide were graded, and the results have therefore been combined in a single group. In table 2 it may be seen that the weight of the testes in the rats given nicotinamide averaged only 1.48 gm., as against 3.09 gm. for rats dosed with α-tocopherol. Since the final body weights

FIG. 1. Uterine pigmentation. (Left) Undosed rat (table 1, No. 18). (Center) Rat dosed with isoniazid (table 1, No. 48). (Right) Rat dosed with DL-α-tocopheryl acetate (table 1, No. 31). Note that the degree of pigmentation in the animal dosed with isoniazid is less than in the undosed animal.

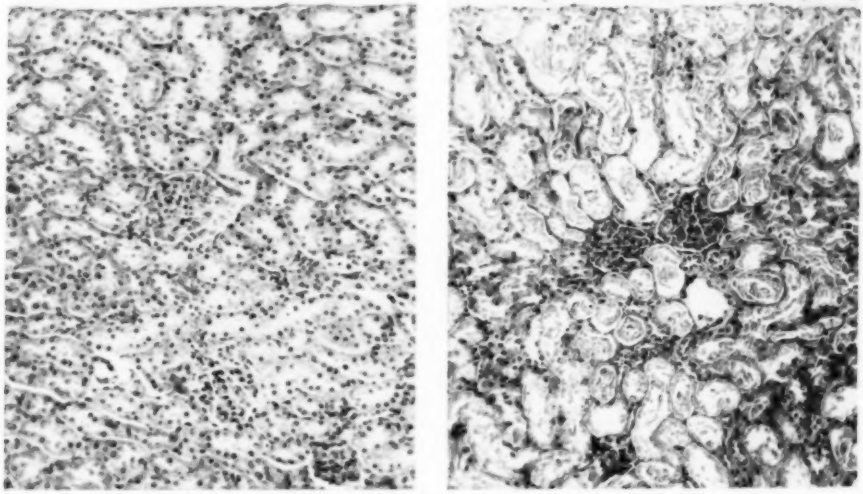

FIG. 2. Photomicrographs of sections of the kidneys of rats fixed three hours after death. (Left) Rat dosed with isoniazid (table 1, No. 39, sex M); histolysis, grade 0. (Right) Undosed rat; histolysis grade 2.5 (table 1, No. 6, sex M).

were also low in the rats given nicotinamide, however, the small size of the testes did not necessarily imply that they were abnormal. Sections were therefore prepared which clearly indicated that most of the tubules had degenerated.

The nicotinamide appeared to have greater potency against brown coloration of the uterus, for which the average marking was 1.2 with nicotinamide as compared with 3.0 in the undosed group. Again growth increases were slow in the rats given nicotinamide.

Teeth: The inclusion of albino rats in this experiment facilitated observations on dental depigmentation. Complete or partial bleaching of the teeth was seen in all rats of this breed which were either undosed or were given nicotinamide, whereas the teeth were normal in the rats given α-tocopherol. Of the piebald rats, 3 of 4 undosed rats had mottled teeth, as compared with 1 of 6 of the rats given nicotinamide, and 0 of 4 of the rats given α-tocopherol. On the evidence of the albinos, nicotinamide had no vitamin E activity. From the observations on the piebalds, no valid conclusion can be drawn.

Isoniazid Dosage with the Diet Containing Cod-Liver Oil

In two series of experiments isoniazid was included in the diet containing cod-liver oil at levels of 0.15 and 0.2 per cent respectively.

Hemolysis: In 4 rats which received 0.2 per

TABLE 2

NICOTINAMIDE DOSAGE. OBSERVATIONS UPON RATS FED UPON A DIET CONTAINING LARD

Undosed Rats				Rats Dosed with α-Tocopherol				Rats Dosed with Nicotinamide				
Number and Breed	Days on Diet	Body Weight Gain	Weight of Testes (gm.) or Uterus Marking	Number and Breed	Days on Diet	Body Weight Gain	Weight of Testes (gm.) or Uterus Marking	Number and Breed	Per Cent of Drug	Days on Diet	Body Weight Gain	Weight of Testes (gm.) or Uterus Marking
♂		gm.		♂		gm.		♂			gm.	
53	121	200	1.14	61	121	268	2.80	67	2.0	121	117	0.88
54	121	192	1.00									
55 A	129	203	2.31	62 A	129	189	3.03	68 A	2.0	129	68	1.21
56 A	129	240	2.04	63 A	129	282	3.43	69 A	2.0	129	79	1.29
		—	—			—	—	70	1.0	121	205	1.55
Average		209	1.62			246	3.09	71 A	1.0	129	116	1.70
								72 A	1.0	129	147	1.82
								73	0.5	121	180	1.49
								74 A	0.5	129	91	1.48
								75 A	0.5	129	189	1.91
											—	—
Average											132	1.48
♀				♀				♀				
57	121	127	3.0	64	121	162	0	76 A	2.0	113	72	0.0
58	121	120	3.0	65	121	212	0	77 A	2.0	129	49	1.0
59 A	129	108	3.0	66	121	104	0	78 A	2.0	129	64	1.0
60 A	129	126	3.0			—		79	1.0	121	65	1.5
		—				159		80 A	1.0	129	71	1.5
Average		120						81 A	1.0	129	94	1.5
								82	0.5	121	48	1.0
								83	0.5	121	78	2.0
								84 A	0.5	129	61	1.0
											—	—
Average											67	1.2

TABLE 3

ISONIAZID DOSAGE. OBSERVATIONS ON RATS FED UPON A DIET CONTAINING COD-LIVER OIL

Undosed Rats					Rats Dosed with α-Tocopherol					Rats Dosed with Isoniazid					
Number	Days on Diet	Body Weight Gain	Color of Fat (Average Marking)	Weight of Testes (gm.) or Uterus Marking	Number	Days on Diet	Body Weight Gain	Color of Fat (Average Marking)	Weight of Testes (gm.) or Uterus Marking	Number	Per Cent of Drug	Days on Diet	Body Weight Gain	Color of Fat (Average Marking)	Weight of Testes (gm.) or Uterus Marking
♂		gm.			♂		gm.			♂			gm.		
85	128	213	4.0	2.21	96	128	253	0	2.46	100	0.15	111	183	0	2.74
86	128	230	4.0	2.36	97	143	336	0.5	2.53	101	0.2	141	123	1.3	2.14
87	128	182	3.7	2.68						102	0.2	141	175	1.7	2.05
88	143	212	4.0	1.20											
89	143	238	3.7	2.14											
		—	—	—			—	—	—			—	—	—	—
Average...		215	3.9	2.12			295	0.3	2.50			131	160	1.0	2.31
♀					♀					♀					
90	99	150	2.7	0	98	111	155	0.3	0	103	0.15	99	106	1	0
91	99	124	3.3	0	99	111	126	0.3	0	104	0.15	99	152	1	0
92	99	142	1.0	0						105	0.2	141	99	0.5	0
93	99	136	3.3	0						106	0.2	141	110	0.2	0
94	143	135	1.5	1											
95	143	130	1.7	1											
		—	—	—			—	—	—				—	—	—
Average...		136	2.3	0.3			142	0.3	0				117	0.7	0

cent of isoniazid, percentages of hemolysis of 66, 81, and 91 were observed. For 4 undosed animals given the same diet, the percentages were 56, 79, 79, and 81, as contrasted with 4 and 6 for 2 rats dosed with α-tocopherol. Again there was no evidence that isoniazid had any vitamin E activity.

Testes and uterus: In table 3, which combines the results for both series of experiments, it may be seen that the testes closely approached the normal size or that the uterus was normal in color in the rats which were dosed with isoniazid. The same normality, however, was observed in most of the undosed animals.

Body fat: For simplicity the markings for the brown color of omental, subcutaneous, and peritesticular or peri-uterine body fats have been averaged in table 3 to give a single reading for each animal. On this criterion isoniazid appeared to have some potency in reducing the intensity of the brown color. Thus, for undosed rats the average brown markings were 3.9 and 2.3 for males and females, respectively. For rats dosed with isoniazid, the averages were 1.0 and 0.7; and for rats dosed with α-tocopherol, 0.3 and 0.3.

Teeth: In this experiment the incisors were normal in all of the rats given isoniazid, but were also normal in 10 of 11 undosed animals. No conclusion can therefore be drawn about the vitamin E potency of the drug.

Discussion

The results of these experiments confirm, although to a limited degree, the claim of Bergel that isoniazid can serve as a substitute for vitamin E. When a basal diet containing cod-liver oil was used, as in Bergel's work, the administration of isoniazid reduced the intensity of brown coloration in the fat deposits of the rats. With this diet, however, no opportunity was given for the drug to simulate vitamin E further in protecting the testes and uterus, since no lesions were observed when the basal diet was given alone. Presumably the amount of vitamin E present in the oil was sufficient to prevent abnormalities in these organs, at least over the period of the experiment. In confirmation of the writers' previous experience (14*a*), no protection was afforded by the cod-liver oil in the hemolysis test, and the abnormal liability to hemolysis remained even when isoniazid was given.

In the present experiments with a diet containing lard, isoniazid appeared to give some protection against brown coloration of the uterus and dental depigmentation. It was also highly potent in preventing post-mortem histolysis in the kidneys. In the hemolysis test and in protecting the testes, however, the drug was ineffective. With lard in the diet the question of protection against brown body fat did not arise.

The ability of isoniazid to prevent some, but not all, of the symptoms of avitaminosis E is not surprising, since similar findings have been made regarding many other substitutes for the vitamin (23). For convenience, the observations of the writers are recapitulated in table 4.

Nicotinamide, when included in the diet containing lard, resembled isoniazid in giving some protection against brown uterus, but was ineffective in the hemolysis test and against degeneration of the testes. Observations on the teeth could not be compared with those of isoniazid, because most of the rats were albinos. In this species, however, nicotinamide clearly gave no protection against dental depigmentation. At the levels tested, body weight increases were retarded. The question arises whether this factor may have exerted an unspecific effect on the rapidity of color development in the uterus. The same question might be raised in regard to isoniazid, although at the levels tested any retardations in weight increase were less obvious and consistent than with nicotinamide. A definite answer on this point cannot be given. In the writers' experience, however, many toxic sub-

TABLE 4

Potency of Isoniazid in Protecting against Various Lesions Typical of Avitaminosis E in the Rat

Abnormality	Fat in Diet	
	Lard	Cod-Liver Oil
Post-mortem renal histolysis	++	NE
Dental mottling in piebald rats	++	NL
Brown uterus	+	NL
Brown body fat	NL	+
Degeneration of testes	0	NL
Hemolysis by dialuric acid	0	0

++ = full protection
+ = partial protection
0 = no protection
NL = no lesion even in undosed rats
NE = not examined

stances, which retard weight increase, are nevertheless ineffective against brown uterus.

In general, the conclusions agree with those of Bergel, at least in supporting the view that isoniazid reduces rather than increases the formation of abnormal brown pigments. If these pigments can be accepted as a sign of peroxidative changes, as is certainly true for the pigment in adipose tissues, it seems logical to conclude that the drug acts as a biologic antioxidant. Activity as an antioxidant upon the fat phase may not be inconsistent with the accumulation of hydrogen peroxide in the aqueous phase. Conceivably, isoniazid might intervene in preventing hydrogen peroxide from expending itself in attacking emulsified fats. It must be emphasized, however, that whatever its mode of action may be, isoniazid is much less potent than certain other vitamin E substitutes in giving protection against abnormal brown colorations. Thus, in both the uterus and body fat, methylene blue can give complete protection, as compared with only partial protection given by isoniazid. It therefore seems unlikely, unless methylene blue has unsuspected therapeutic powers, that the value of isoniazid against the tubercle bacillus depends intrinsically on its potency as an antioxidant.

Summary

In rats given a standard diet deficient in vitamin E, with lard as its fat component, prophylactic dosage with isoniazid was beneficial against some of the lesions typical of avitaminosis E, but not against others. Thus, the drug prevented the rapid post-mortem histolysis in the kidneys and mottling of the incisor teeth. It also reduced the intensity of the abnormal brown coloration in the uterus. Against degeneration of the testes and hemolysis *in vitro* by dialuric acid, isoniazid was ineffective.

With the same diet, nicotinamide also slightly reduced the intensity of brown coloration in the uterus. No protection was given against degeneration of the testes or in the hemolysis test.

In rats given a similar diet, which contained cod-liver oil in place of lard, isoniazid reduced the intensity of the abnormal brown color of the body fat, which is caused by the oil when the vitamin E intake is inadequate. The drug was ineffective in the hemolysis test. The experiments were not continued long enough for brown coloration of the uterus or degeneration of the testes to develop in the undosed animals.

These findings confirm, although only to a limited degree, the claim of Bergel that isoniazid can act as a substitute for vitamin E. In this role it must reduce, rather than augment, the rate of peroxide formation in highly unsaturated components of the body fat.

In preventing some of the lesions typical of avitaminosis E but not others, isoniazid resembles many other artificial substitutes for the vitamin. In the experience of the writers it is less potent than methylene blue, which gives complete protection against brown coloration in the uterus and body fat.

Sumario

La Medicación con Isoniacida en las Ratas Deficientes en Vitamina E

En las ratas alimentadas con un régimen estándard deficiente en vitamina E, con manteca de cerdo como componente lípido, la medicación profilàctica con isoniacida resultó beneficiosa contra algunas de las lesiones típicas de la avitaminosis E, pero no contra otras. Por ejemplo, la droga impidió la rápida histólisis postmortal en los riñones y el moteamiento de los dientes incisivos, reduciendo también la anómala pigmentación parda del útero. Contra la degeneración de los testículos y la hemólisis *in vitro* producida por el ácido dialúrico, la isoniacida resultó ineficaz.

Con el mismo régimen dietético, la nicotinamida también rebajó ligeramente la intensidad de la pigmentación parda en el útero, pero tampoco suministró protección contra la degeneración testicular o en la prueba de la hemólisis.

En las ratas a las que se suministró un régimen semejante, pero que contenía aceite de hígado de bacalao en vez de manteca, la isoniacida redujo la intensidad del anómalo color pardo de la grasa orgánica, que produce el aceite cuando la ingestión de vitamina E es inadecuada. La droga se mostró ineficaz en la prueba de la hemólisis. No se continuaron los experimentos por tiempo sufficiente para que aparecieran pigmentación parda del útero o degeneración testicular en los animales no tratados.

Estos hallazgos confirman, aunque sóloen forma limitada, el aserto de Bergel, de que la isoniacida puede obrar como substituto de la vitamina E. En esta capacidad, debería rebajar, más bien que aumentar, la velocidad de la formación de peróxido en los componentes sumamente insaturados de la grasa del cuerpo.

En la prevención de algunas, pero no de otras, de las lesiones típicas de la avitaminosis E, la isoniacida se parece a otros muchos substitutos artificiales de dicha vitamina. Según lo observado

por los AA., es menos potente que el azul de metileno, que suministra protección absoluta contra la coloración parda del útero y de la grasa del cuerpo.

Resume

Administration de l'isoniazide chez des rats carencés en vitamine E

Chez des rats recevant une ration standard déficiente en vitamine E, le lard représentant l'élément graisse, l'administration prophylactique d'isoniazide s'est montrée favorable contre certaines des lésions caractéristiques de l'avitaminose E., mais non contre les autres. C'est ainsi que la drogue prévenait l'histolyse rapide post-mortem des tissus rénaux et les taches de l'émail des incisives. Elle réduisait également l'intensité de la coloration brune dans l'utérus. L'isoniazide s'est révélée inefficace contre la dégénerescence des testicules et l'hémolyse *in vitro* causées par l'acide dialurique. La ration étant la même, l'administration de nicotinamide réduisait aussi légèrement l'intensité de la coloration brune dans l'utérus, mais elle était sans effet sur l'épreuve de l'hémolyse.

Chez les rats recevant une diète similaire, contenant de l'huile de foie de morue au lieu de lard, l'isoniazide réduisait l'intensité de la couleur brune anormale des graisses organiques, qui est due à l'huile quand l'apport en vitamine E est inadéquat. Cette drogue était inefficace dans le test d'hémolyse. L'expérimentation n'a pas été poursuivie assez longtemps pour que la coloration brune de l'utérus ou la dégénerescence des testicules soit observée chez les animaux non traités.

Bien qu'à un degré limité, ces résultats confirment le postulat de Bergel, qui admet que l'isoniazide peut agir comme substance de remplacement de la vitamine E. Dans ce rôle elle tend à réduire plutôt qu'à augmenter la vitesse de formation du peroxyde dans les composants polyéthyléniques des graisses organiques.

En ce qui concerne la prévention de certaines des lésions caractéristiques de l'avitaminose E, mais non des autres, l'isoniazide ressemble à de nombreux autres substituts artificiels de la vitamine E.

Selon l'expérience des auteurs, l'isoniazide est moins active que le bleu de méthylène qui donne une protection complète contre la coloration brune de l'utérus et des graisses organiques.

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A TEMPERATE BACTERIOPHAGE FROM MYCOBACTERIUM BUTYRICUM[1, 2]

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INTRODUCTION

In 1954 Hnatko (1) found a strain of Mycobacterium carrying a temperate phage. In the following year, Smith (2) was unable to demonstrate any lysogenic organisms among 34 mycobacteria, including cold-blooded, bovine, and human types of tubercle bacilli. Russell and associates (3) also have reported failure to demonstrate lysogeny in their mycobacterial stock cultures. In previous studies, lysis (4, 5) of mammalian tubercle bacilli (H37Rv) was observed after eight to ten days' growth in nitrogen-deficient medium and also subsequent to the action of various inducing agents. This lysis conceivably might result from a lysogenic state of the tubercle bacilli; however, the present writers have been unable by a variety of techniques to isolate a temperate phage from H37Rv.

A search for lysogenic strains among other types of tubercle bacilli revealed *Mycobacterium butyricum* (6) to be naturally lysogenic, i.e., it transmits hereditarily the power to produce bacteriophage (7). The present study describes the phage-host relationship on lysogenic *M. butyricum*. Similar determinations were made on the temperate phage of *M. butyricum* and its indicator strain, *Mycobacterium tuberculosis*, ATCC No. 607.

MATERIALS AND METHODS

Mycobacterium butyricum: A desiccated culture was obtained from Dr. Martin Frobisher in January, 1949. Since that time, it has been propagated on egg medium with transfers made every three to six months. In 1955 a subculture was made by three successive single-colony isolations on nutrient agar, and subsequently it has been carried on nutrient-agar slants.

Indicator bacteria: A strain of *M. tuberculosis* ATCC No. 607[3] was used as indicator. It was isolated from a culture labeled F21 (8) received from Dr. Seymour Froman. A broth suspension was made from the original culture and streaked on nutrient-agar medium. Two colony types appeared in about equal numbers and were designated 607*B* (for buff) and 607*W* (for white). The former was used as the indicator strain. Both colony types support development of the temperate phage produced by *M. butyricum*. A streptomycin-resistant strain of 607, one of the stock cultures used in this laboratory, was used in the streptomycin technique (*see* below).

The methods used for growing the organisms in stock cultures and for experimental purposes have been given in detail elsewhere (9).

Media: Broth consisted of 0.8 per cent nutrient broth (Difco) and 0.5 per cent sodium chloride in distilled water. The pH was adjusted to 7.0 with 10 per cent sodium hydroxide. Tween 80[4] to 0.06 per cent was added to the broth for growing lysogenic *M. butyricum*.

Phage production and purification: The phage from *M. butyricum* produced either spontaneously or after induction by ultraviolet irradiation was designated R1, and the parent strain, therefore, as *M. butyricum(R1)*. The phage suspension used in these studies was prepared from a single plaque resulting from plating *M. butyricum(R1)* on the indicator strain, 607B. After three successive single-plaque isolations, a phage stock was obtained by elution in broth of confluent-lysis plates (9). The phage was filtered through 02 Selas filter funnels and centrifuged alternately three times at 40,000 r.p.m. for ninety minutes and at 3,000 r.p.m. for fifteen minutes. The final preparation had an infectious titer of 1×10^{12} plaque-formers per milliliter.

Phage and bacterial assays: Phage and bacterial assays were made on nutrient agar by the soft-agar technique. The formulas of the media were the same as reported previously (9) except for the addition of 10^{-3} M calcium chloride to both the bottom and soft-agar layers. Either 0.1 ml. or 0.05 ml. of a suitable dilution of the phage stock was mixed with five drops of indicator bacteria (5×10^7 colony-formers per ml.) in the melted soft-agar prior to plating for plaque development. For the streptomycin technique (10), 20 γ of streptomycin per ml. were added to the bottom-agar and to the soft-agar prior to autoclaving (11).

The bacterial assays were made by diluting the

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[2] The experimental results on which this paper is based were presented at the meeting of the Society of American Bacteriologists in Chicago on April 29, 1958.

[3] The strain of 607 used here (607*B*) is not the same strain as the strain of 607 used in experiments reported by Bowman (9).

[4] Polyoxyethylene sorbitan monooleate, Atlas Powder Co.

bacterial suspensions so that by using the soft-agar technique 0.05 ml. of the final suspension gave approximately 250 colonies when plated and incubated.

The ultraviolet irradiations: This procedure was performed with a Westinghouse sterilamp supplied by a Sola constant-voltage transformer. Samples were irradiated in a layer 1 to 2 mm. thick at a distance of 50 cm. from the center of the lamp. The intensity at the sample was about 9.5 ergs per sec. per mm². This dose inactivates 99 per cent of *M. butyricum(R1)* in 120 seconds.

Electron microscopy[5]*:* Purified, high-titer suspensions of R1 centrifuged and resuspended in 1 per cent ammonium carbonate (pH 7.0) were deposited on carbon-covered grids by a modified spray technique of Backus and Williams (12). The specimens were shadowed with platinum at an angle of $\tan^{-1} \frac{1}{3}$, and then examined with an RCA, EMU-2 electron microscope.

Results

Growth of M. butyricum(R1) and spontaneous production of phage R1: Cultures of *M. butyricum (R1)* were initiated by diluting primary cultures (9) 1:200 in Tween-broth and incubating at 37° C. with aeration. Under these conditions the logarithmic phase of growth began after about an eight-hour lag period and was maintained for sixteen hours. After twenty-two to twenty-four hours of total incubation, the growth rate began to decline toward the stationary phase.

The relationship between the growth of *M. butyricum(R1)* and spontaneous production of phage R1 was investigated, but the results could not be quantitated accurately because of the low production of phage. The ratio of free phage to colony-formers in log-phase aerated cultures of *M. butyricum(R1)* is about 10^{-6}. From colonies derived by diluting and streaking *M. butyricum(R1)* on nutrient agar, it was shown that 100 per cent (25 of 25 isolates) of such colonies gave rise to bacterial clones capable of producing phage. These results indicate that the reason for the low ratio of phage to bacteria is not due to an inhomogeneity of the bacterial strain with respect to the fraction of cells lysogenic for phage R1.

Induction of M. butyricum (R1) by ultraviolet irradiation: Cells for irradiation were harvested by centrifugation after sixteen and after twenty-four hours of incubation. The centrifuged pellet was suspended in M/15 phosphate buffer containing 0.06 per cent Tween 80 (pH 7.0) in a volume equal to that of the original culture. These preparations were irradiated with ultraviolet light. At various times during the irradiation, samples were removed and aliquots were suitably diluted in broth and plated for colony-formers and for plaque-formers. The results are shown in figure 1.

The solid upper curves indicate only a slight effect of ultraviolet light on colony formation at low dosage. After approximately sixty seconds the fraction of colony-formers appears to decrease exponentially. The lower curves in figure 1 show that the fraction of induced bacteria producing plaques increases with the dose, reaches a maximum at sixty seconds, and then decreases. No significant difference was noted between the sixteen- and the twenty-four-hour cells with respect to the surviving fractions forming colonies or with respect to plaque formation. This experiment indicates that only about 0.01 per cent of *M. butyricum(R1)* is inducible, and that sixteen- and twenty-four-hour cells are equally inducible.

Incubation with aeration in Tween-broth of ultraviolet-irradiated cells does not lead to a visible clearing of the culture, but leads to an increase in the optical density because of multiplication of the surviving bacilli.

Phage development after induction: Cells of log-phase *M. butyricum(R1)* were irradiated in Tween-buffer with an optimal dose of ultraviolet light (*see* figure 1). After irradiation, aliquots were diluted into broth containing various reagents (*see* table 1) and incubated. Samples were taken at intervals and plated for total infectious centers and for free phage. The streptomycin technique (10) was used to assay the latter. The technique is based on the findings that free phage forms plaques on streptomycin-resistant indicator bacteria in the presence of streptomycin. Lysogenic bacteria (susceptible to streptomycin) do not grow in the presence of streptomycin and, therefore, the prophage does not have an opportunity to become vegetative phage (recognized by plaque production). However, beyond a certain stage of phage development in a lysogenic cell, the plaque-forming ability is unaffected by streptomycin, and those cells which have reached or progressed beyond this point also are registered as plaques in the presence of streptomycin.

The results given in table 1 show that the minimal latent period (time between dilution of irradiated bacteria into growth medium and first measureable increase in plaque count) was 360 minutes. The rise period was about 120 minutes. The average number of phage particles liberated

[5] The micrographs were prepared by Mr. John Brown, Optical Department, Engineering Experiment Station, Georgia Institute of Technology, Atlanta, Georgia.

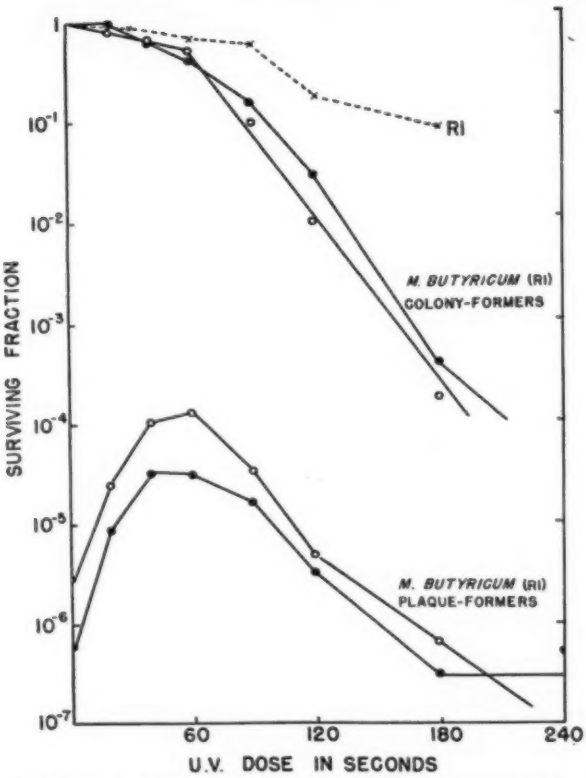

FIG. 1. Ultraviolet induction of *Mycobacterium butyricum(R1)* in Tween-buffer. After sixteen and after twenty-four hours of growth, log-phase cells were transferred to Tween-buffer (1:1) and irradiated. Input bacterial concentrations: 16-hour cells (open circles) = 6.03×10^7 colony-formers per ml.; 24-hour cells (closed circles) = 2.4×10^8 colony-formers per ml. Samples were taken at intervals and after irradiation were plated immediately for infectious centers. The surviving bacteria (colony-formers) were assayed simultaneously. The broken curve shows the susceptibility to ultraviolet irradiation of R1 particles suspended in Tween-buffer.

per original plaque-forming bacterium ranged from 4 to 20.

Infection of indicator bacteria with phage R1: The 607B-R1 system was studied with respect to adsorption rate constant and one-step growth characteristics. It was found that in broth, with or without calcium ion supplement, at an input multiplicity of about 0.1, phage R1 adsorbs exponentially to log-phase cells of 607B (indicator strain). The adsorption rate constant is approximately 3×10^{-10} ml. min.$^{-1}$ colony-former^{-1}. The one-step growth experiments showed that the minimal latent period is about 240 minutes and that the average burst size is about 120 when the broth is supplemented with 10^{-3} M calcium chloride.

When free phages of R1 were plated with the indicator bacteria, 607B, plaques developed in twenty-four to forty-eight hours. If a large number (5×10^8 colony-formers per plate) of plating bacteria was used, plaques developed during twenty-four hours of incubation but, because of the relatively low adsorption rate constant (*see* above) and the long latent period for R1 (on 607B), the size of the plaques at the end of bacterial growth was small. However, when the bacterial inoculum was diluted 1:10, approximately forty-eight hours of incubation were re-

TABLE 1

CHARACTERISTICS OF TEMPERATE PHAGE R1*

Media†	Minimal Latent Period	Rise Period	Average Burst Size
	minutes	*minutes*	
Broth	360	Ca. 120	4
Tween-broth	360	Ca. 120	8
Tween-broth with 1% bovine albumin‡	330	Ca. 120	13
Tween-broth with 1% bovine albumin‡ with M/0.001 Ca^{++} supplement	360	Ca. 120	19

* Data obtained from *M. butyricum(R1)* UV induced in Tween-buffer

† The moment of transfer of ultraviolet-induced cells to these media established the zero time for the growth experiment

‡ Heat inactivated, 56° C. for 30 minutes

quired for attaining about the same density of the bacterial film, and the resulting plaques were larger (figure 2).

Plaques are clear when they originate from free phages of R1 plated on 607B. On the other hand, R1 plaques originating from plaque-producing cells of *M. butyricum(R1)* vary in degree of turbidity. The turbidity results from the growth of non-plaque-producing cells of *M. butyricum(R1)* on the plate along with the indicator bacteria. The degree of turbidity is dependent on the ratio of phage-producing to non-phage-producing cells within the culture. A low ratio (10^{-6}) results in a turbid plaque, while a higher ratio (10^{-4}, obtained by ultraviolet irradiation) gives less turbid plaques.

Effect of manganese chloride on plaque production: The virus yields from phage-infected bac-

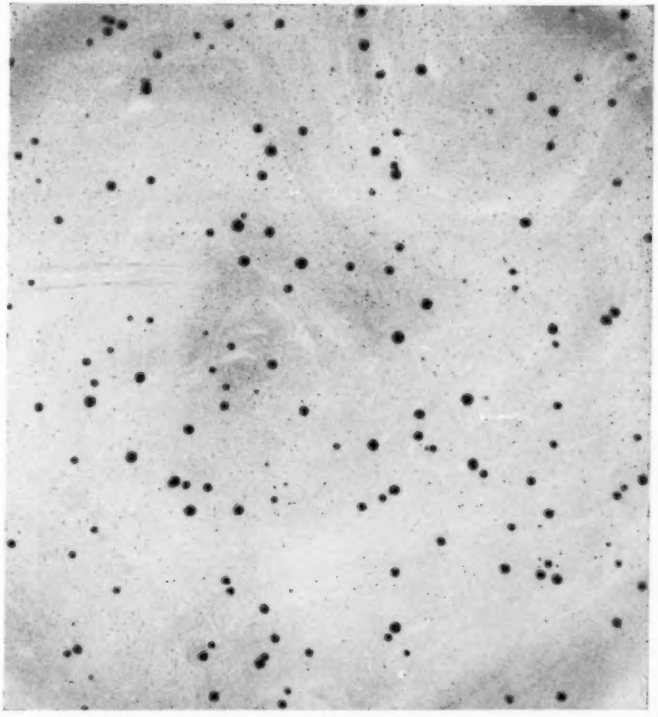

FIG. 2. Plaques of phage R1 on indicator strain 607*B*. Source of infectious centers: log-phase *M. butyricum(R1)* induced with an optimal dose of ultraviolet in Tween-buffer, transferred to growth medium and incubated for eight hours (two hours longer than the minimal latent period); then an aliquot plated and incubated 48 hours at 37° C. for plaque development.

teria are markedly influenced by the mono- or divalent cation content of the medium. Huybers (13) working with another lysogenic system showed manganese to be necessary for phage production.

In a preliminary experiment, 0.1 ml. of 1M $MnCl_2$ was added to the melted soft-agar just prior to inoculating with ultraviolet-induced *M. butyricum(R1)* and indicator bacteria. After twenty-four hours' incubation the number of plaques produced was increased about five times.

TABLE 2

EFFECT OF PREINCUBATING* *M. BUTYRICUM(R1)* WITH MANGANESE ON PLAQUE PRODUCTION (Figures Indicate Total Plaque Counts)

	Treatment after Ultraviolet Irradiation			
	None	1 Hour with 10^{-1} M Mn^{++}-Supplemented Broth	1 Hour with 10^{-2} M Mn^{++}-Supplemented Broth	1 Hour with 10^{-3} M Mn^{++}-Supplemented Broth
No ultraviolet	8	13	12	11
Ultraviolet four minutes in broth	226	210	195	278

* At 25° C. in dark

Subsequently, a log-phase culture of *M. butyricum (R1)* was divided into two parts, one of which was induced with an optimal dose of ultraviolet light (in Tween-broth). The other was not irradiated. Manganese was added to 0.9-ml. aliquots of each preparation so that the final concentration of the supplemental ion varied from zero to 10^{-3} M. The aliquots with no manganese supplement were assayed for total phage at zero time, and the remaining tubes were incubated at room temperature (approximately 25° C.) in the dark for one hour and then assayed for total phage.

The results shown in table 2 indicate that contact with manganese for one hour was not sufficient to elicit the effects observed in the preliminary experiments.

Host range of phage R1: Included within the host range of R1 are 607*W* and *M. ranae*. The efficiency of plating on these hosts is 2.3 and 1.0 respectively. Another strain of 607 (9) does not support phage growth.

Plaques of R1 do not develop on H37Rv, but Tween-grown H37Rv after treatment with broth at 4° C. for twenty-four hours allows phage attachment. About 50 per cent of the phage is adsorbed (input multiplicity, $m = 25$) during thirty minutes at 37° C. At this multiplicity (actual $m = 12.5$), according to the Poisson distribution, one expects 100 per cent infection.

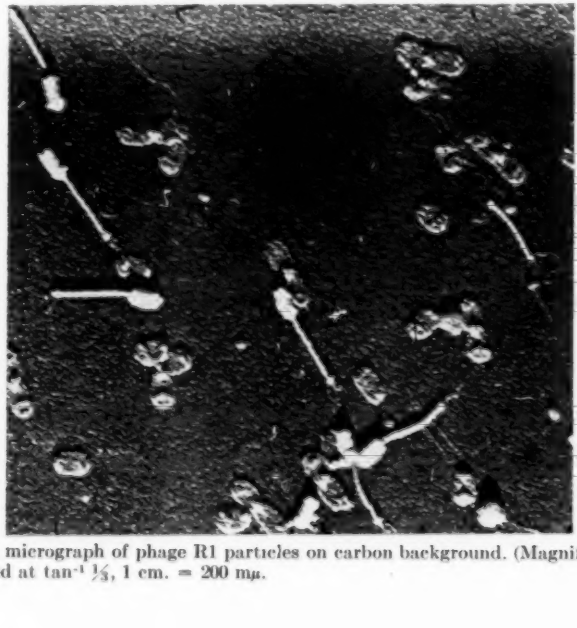

FIG. 3. Electron micrograph of phage R1 particles on carbon background. (Magnification: 44,000 ×) Platinum shadowed at $\tan^{-1} \frac{1}{5}$, 1 cm. = 200 mμ.

The results showed no loss of colony-formers after adsorption. Forty single-colony isolates were made and tested for lysogeny. None of the isolates were found to be lysogenic.

Immunity: When free phages of R1 are plated on *M. butyricum(R1)*, plaques do not develop. Adsorption experiments, using Tween-grown *M. butyricum(R1)*, which were treated overnight in broth at 4° C., showed that such cells did not adsorb phage R1.

Electron micrographs: A modification of the spray technique of Backus and Williams (12) was used. Figure 3 is an electron micrograph of R1 particles on carbon background. The heads are approximately 100 by 60 mμ., and the tails are about 200 by 20 mμ. The particles of R1 are about 300 mμ. long. Although the heads appear to be cylindrical, protuberances observed on many strongly suggest that the heads are not cylindrical but are hexagonal in shape. Sharp angles in the outlines of the shadows lead to the same conclusions.

Discussion

The development of phage R1 after induction of lysogenic cells or after infection of susceptible cells differs in two respects: the latent period, and the burst size. The latent period of *R1*-infected cells of the indicator strain is about four hours, whereas the latent period of ultraviolet-induced lysogenic cells is about six hours. Such phenomenon (different lengths of latent period) is not unusual in other lysogenic systems (14). However, to the present writers' knowledge, no latent period, for either temperate or virulent phages, of four to six hours has been reported. The average burst size for ultraviolet-induced *M. butyricum(R1)* was shown to be about 20 when assayed on 607*B*. The burst size of *R1* on singly infected cells of 607*B* is about 120. It has been suggested by Dr. G. Bertani that host-controlled variation (15) could account for the apparent low yield of phage upon lysis of *M. butyricum (R1)*. Another explanation for the observed difference in the burst sizes is that more suitable physiologic conditions for phage maturation exist in cells of 607*B* than in *M. butyricum(R1)*. It has been shown by Huybers (13) that with *Bacillus megaterium* a deficiency in manganese prevents phage development (after induction), but that, if Mn^{++} is restored within a suitable time after ultraviolet treatment, normal phage production is obtained. Preliminary experiments have shown that addition of manganese ($MnCl_2$) to the plating medium increases the plaque count by a factor of about 5. It has not been determined whether the increased plaque count is due to an increased yield of phage per bacterium or whether the increased yield is due to a "recovery" of plaques which are not formed in the absence of manganese. Both factors could operate to give the increased plaque count.

Although it was found that sixteen-hour and twenty-four-hour log-phase cells lysogenic for *R1* were equally inducible, the fraction of colony-formers yielding phage after induction is very low (about 0.0001) in either case. The magnitude of this fraction is about 100 times larger than the fraction of colony-formers which produces phage spontaneously. This estimate of the fraction of colony-formers capable of producing phage, both before and after ultraviolet irradiation, might be significantly altered if the bacterial suspension consisted of a completely dispersed state, i.e., if the colony-forming units consisted of single cells.

According to Bertani (14), readsorption of the homologous phage by cells in a lysogenic culture does not lead to lysis. The lysogenic cells are therefore immune to superinfection by the homologous phage. In a few instances the homologous phage is not adsorbed by the lysogenic bacteria (14). The results of adsorption studies with *R1* on *M. butyricum(R1)* appear to fit the latter situation. Adsorption studies made on diphtherial and mycobacterial phage-host systems (9, 16, 17) have indicated that Tween 80 inhibited adsorption. As yet, it has not been determined whether Tween plays a similar role in adsorption of *R1* on *M. butyricum(R1)*.

It would appear that the low phage content of cultures of *M. butyricum(R1)*, both before and after ultraviolet treatment, is not due to progeny phage adsorbing to unlysed bacteria or to bacterial products (phage-receptor sites).

Strain H37Rv does not support plaque production in plating experiments; yet, in adsorption studies, it was found that some phages adsorbed to H37Rv ($m = 12.5$). To account for these discrepant results several reasons are given: (*a*) desorption of reversibly bound phage occurred subsequent to the assays for supernatant phage; (*b*) strain H37Rv could be naturally lysogenic (*see* Introduction) with a phage closely related to

R1, so that immunity is conferred on H37Rv; or (*c*) since H37Rv is present in clumps, it is conceivable that *R1* adsorbed to some of the bacillary clump members and that surviving members were able to form colonies. At present, data are not available to indicate which of the above, or other reasons, may account for the failure of adsorbed phage to prevent colony formation.

The electron micrographs of fresh lysates of *R1* reveal that the particles are larger than particles of D29 (18), or its plaque-type variants, D29*L* and D29*S* (9). In addition, it appears that the heads of *R1* are cylindrical, whereas D29 heads appear round. Phage *R1* apparently is larger than many other temperate phages (14).

Summary

A laboratory strain of *Mycobacterium butyricum* has been demonstrated to be naturally lysogenic. The phage has been isolated and termed *R1*. The rate of spontaneous phage production is too low to quantitate accurately. Various reasons for low phage production are discussed.

The lysogenic organisms are inducible with ultraviolet irradiation, and the fraction of colony-formers yielding phage after an optimal dose of ultraviolet is about 10^{-4}. The latent period (after ultraviolet induction) is about six hours, and the burst size is approximately 20. Single infection of the susceptible strain, 607*B*, with the temperate phage leads to a latent period of about four hours and an average burst size of 120.

The organisms lysogenic for *R1* appear not to adsorb the homologous phage.

Electron micrographs of *R1* particles show that the phage is about 300 mμ. long and that the heads are probably elongated hexagons.

Sumario

Un Bacteriófago Templado Obtenido del Mycobacterium Butyricum

Se ha demostrado que una cepa de laboratorio del *Mycobacterium butyricum* is lisógena naturalmente. Se ha aislado el bacteriófago del mismo, denominándolo R1. La bacteriofagogenia espontánea es demasiado lenta para medición cuantitativa exacta. Se discuten varias razones para la baja fagogenia.

Los microbios lisógenos son inducibles con la irradiación ultravioleta y la fracción de colonizadores que producen bacteriófago después de una dosis óptima de rayos ultraviolados es de unos 10^{-4}. El período de latencia (después de la inducción ultravioleta) es de unas seis horas y el tamaño al reventar es aproximadamente de 20. La infección aislada de la cepa sensible, 607B, por el bacteriófago templado conduce a un período latente de unas cuatro horas y el tamaño al reventar promedia 120.

Los microbios lisógenos para el R1 no parecen adsorber el bacteriófago homólogo.

Las electronomicrografías de las partículas del R1 revelan que el bacteriófago mide unas mμ de largo y que las cabezas son probablemente hexágonos alargados.

Resume

Un bactériophage atténué provenant du Mycobacterium butyricum

Une souche de *Mycobacterium butyricum* de laboratoire s'est révélée naturellement lysogénique. Le phage a été isolé et dénommé R1. La vitesse de production spontanée du phage est trop faible pour l'évaluer avec précision. Les diverses raisons de cette faible production du phage sont commentées.

Les organismes lysogéniques peuvent être engendrés par l'irradiation aux ultra-violets, et la fraction des organismes faisant partie des colonies produisant des phages à la suite d'une dose d'ultra-violets optima est d'environ 10^{-4}. La période latente (après l'exposition aux ultra-violets) est d'environ six heures; les phages apparaissent au bout de vingt heures environ. Une seule inoculation de la souche sensible, 607B, avec le phage atténué, amène une période latente d'environ quatre heures et une production de phages au bout de 120 heures.

Les organismes lysogéniques pour R1 semble ne pas adsorber le phage homologue.

Les micrographes électroniques de particules R1 montrent que le phage a une longueur d'environ 300 mμ et une tête ressemblant à un hexagone allongé.

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THE MECHANISM OF THE TOXICITY OF CORD FACTOR[1]

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INTRODUCTION

For many years investigators have attempted to demonstrate a bacterial toxin related to virulence in order to account for the pathogenesis of tuberculosis, but always without success. This led to a current view (1–3) that the toxic manifestations of tuberculosis are mainly based on allergic reactions which added little to the understanding of the direct role of toxins in the pathology of tuberculosis.

The isolation of a toxic lipid component from virulent tubercle bacilli by Bloch (4, 5) aroused a great interest in this connection. Although the material first described under the name of "cord factor" was found to be a crude mixture of various lipids (6), a thorough purification by Noll and Bloch (7) yielded a powerful purified toxin, a few micrograms of which exerted a characteristic delayed toxicity for mice. Finally, recent extensive analytical (8–11) and synthetic (12) studies on its chemical structure confirmed that the cord factor is trehalose-6,6′-dimycolate.

The present writers obtained a purified sample of cord factor from Bloch and studied the biologic activity of this substance. It was reported in a previous communication (13) that the toxicity of cord factor for mice was confirmed, but it did not influence the *in vivo* multiplication and survival of either virulent or avirulent human tubercle bacilli. The paper reported also that the intraperitoneal injection of 0.1 mg. of cord factor decreased, approximately twenty-four hours later, the succinic and malic dehydrogenase activity of mouse liver.

It was found in further studies that the injection of cord factor decreased not only the above mentioned enzymic activity, but also the activity of some other diphosphopyridine nucleotide-linked dehydrogenase in mouse liver.

Studies on the mechanism of this inhibition of enzymic activity by cord factor are described in the present paper. The correlation of the phenomenon with the infection of animals by tubercle bacilli is also of great interest. It was studied in parallel with the work reported here and will be described in a subsequent communication.

MATERIALS AND METHODS

Cord factor: One hundred milligrams of purified cord factor were obtained through the courtesy of Dr. Hubert Bloch, Department of Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania. The desired amounts of cord factor were dissolved in 0.1 ml. of olive oil and injected intraperitoneally into mice. For use in the *in vitro* experiment, the material was dissolved in propylene glycol and then suspended in distilled water. Solutions in olive oil or propylene glycol were sterilized by heating at 100°C. for thirty minutes and stocked at 5°C. as 0.1 per cent solution.

Animals: A pure line of Swiss albino mice (strain Na-2) was obtained from a single source. Male animals weighing 10 to 15 gm. at four to six weeks of age were used. They were maintained in groups of 5 to 10 mice in separate metal cages and were fed pellet diet[2] and water *ad libitum*.

Preparation of liver homogenate and mitochondrial fraction: The cord-factor–treated animals were sacrificed by cervical dislocation at the same time as control animals, which received only 0.1 ml. of olive oil. The liver was removed, washed in chilled saline, and 10 per cent homogenate in cold 0.1 M phosphate buffer (pH 7.4) or 0.25 M sucrose was prepared, using a glass homogenizer cooled in an ice-bath.

Mitochondrial fraction was prepared according to a slight modification of the method described by MacFarlane and Datta (14). The mouse liver was homogenized for one minute in the presence of 0.25 M sucrose and centrifuged at 1,000 r.p.m. for 10 minutes. The supernatant was centrifuged at 7,000 r.p.m. for 30 minutes. The precipitate of the centrifugation at 7,000 r.p.m. was collected and resuspended in 0.25 M sucrose, and centrifuged at 2,000 r.p.m. for 5 minutes. The supernatant was again centrifuged at 7,000 r.p.m. for 30 minutes, and the precipitate was suspended in 0.25 M sucrose. The last suspension was used as mitochondrial fraction. All procedures were done at 0°–5°C.

Estimation of dehydrogenase activity: Succinic

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[2] Oriental Pellet Diet MC 5, Oriental Yeast Co., Osaka.

TABLE 1

EFFECT OF CORD FACTOR ON VARIOUS DEHYDROGENASE ACTIVITIES OF MOUSE LIVER

Substrate	Co-Factor of Dehydrogenase	γ of Formazan Reduced per Mg.N per Minute	
		Control	Cord-Factor–treated
Succinate		2.54 ± 1.39	0.80 ± 0.26*
Malate	Diphosphopyride nucleotide (DPN)	0.72 ± 0.21	0.54 ± 0.24*
Lactate	Diphosphopyride nucleotide (DPN)	0.88 ± 0.31	0.40 ± 0.02*
α-Glycerophosphate	Diphosphopyride nucleotide (DPN)	3.86 ± 1.21	2.54 ± 0.41*
Glutamate	Diphosphopyride nucleotide (DPN)	1.26 ± 0.45	1.00 ± 0.34†
Citrate	TPN‡ (DPN)	2.06 ± 1.38	2.96 ± 0.82
Alanine	FAD§	1.24 ± 0.42	1.62 ± 0.25

0.1 mg. of cord factor per 0.1 ml. of olive oil was injected intraperitoneally into 5 mice. Five control animals received 0.1 ml. of olive oil only. The estimation of dehydrogenase activity was done 24 hours after the injection.

The reaction mixture consisted of 1.0–2.0 mg. N of the liver homogenate, 100 μ M of substrate, and final 0.03% TTC in final 0.1 M phosphate buffer (pH 7.4).

Enzymic activity is given as γ formazan reduced per mg. N of liver per minute ± standard deviation.

* Indicates that the difference is significant ($\alpha = 0.01$).

† $\alpha = -0.05$.

‡ Triphosphopyridine nucleotide.

§ Flavine adenine dinucleotide.

dehydrogenase and other dehydrogenase activity of liver homogenate and mitochondrial fraction was measured by the colorimetric method reported by Kun and Aboad (15), using triphenyltetrazolium chloride (TTC) as hydrogen acceptor. Enzyme activity was expressed as γ of formazan reduced per mg.N of liver homogenate per minute ± standard deviation. The significance of the difference between experimental and control animals was examined by statistical analysis.

Succinic dehydrogenase activity was measured also by the manometric method described by Slater (16) and the spectrophotometric assay of Slater and Bonner (17).

Estimation of succinic oxidase and cytochrome oxidase: Succinic oxidase activity of liver homogenate was measured manometrically in the presence of succinate. Cytochrome oxidase activity was determined manometrically by measuring the oxygen uptake in the presence of cytochrome *c* and hydroquinone to reduce the cytochrome *c*.

TABLE 2

RELATION BETWEEN THE DOSES OF CORD FACTOR AND THE DECREASE OF SUCCINIC DEHYDROGENASE ACTIVITY OF MOUSE LIVER

γ of Cord Factor Injected Intraperitoneally	Number of Mice Used	γ of Formazan Reduced per Mg. N per Minute (Mean ± Standard Deviation)
None	5	4.43 ± 1.18
Olive oil, 0.1 ml.	5	4.97 ± 0.48
1	5	4.48 ± 0.83
5	5	2.77 ± 0.50*
10	5	2.50 ± 0.43*
100	5	2.80 ± 0.89*

Cord factor was injected intraperitoneally into mice 24 hours before the determination of enzyme activity, dissolved in 0.1 ml. of olive oil.

* Indicates that the difference is significant ($\alpha = 0.01$).

RESULTS

Effect of Cord Factor on Various Dehydrogenase Systems of Mouse Liver

The rate of reduction of triphenyltetrazolium chloride by the liver homogenate of control and cord factor–treated mice was compared using the following substrates: succinate, lactate, malate, α-glycerophosphate, citrate, glutamate, and alanine.

It is shown in table 1 that the intraperitoneal injection of 0.1 mg. of cord factor lowers the succinic, malic, lactic and α-glycerophosphoric dehydrogenase activity of mouse liver twenty-four hours after the injection. The co-factor of the last three dehydrogenases is diphosphopyridine nucleotide. But the same injection does not affect the reduction of triphenyltetrazolium chloride with citrate, glutamate, and alanine.

The relationship of the intraperitoneal doses of

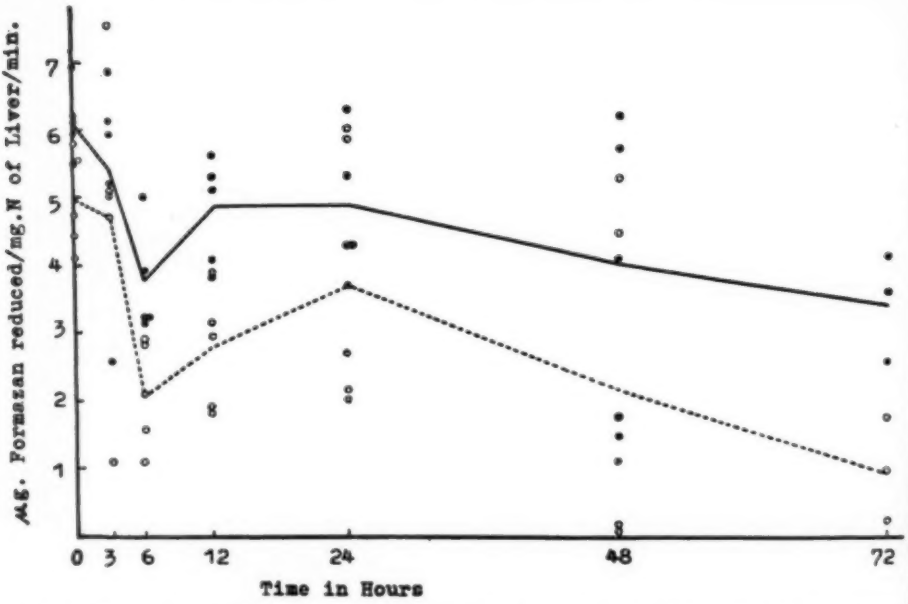

FIG. 1. Decrease of succinic and α-glycerophosphoric dehydrogenase activity after intraperitoneal injection of cord factor: 0.01 mg. of cord factor was injected into mice intraperitoneally at 0 time.
⊹: Succinic dehydrogenase
○: α-Glycerophosphoric dehydrogenase

cord factor and the decrease of liver succinic dehydrogenase activity is shown in table 2. The effective minimum found was 5 γ and there was no parallel, above this amount, between the doses of cord factor and the degree of the lowering of enzyme activity.

When the activity of succinic and α-glycerophosphoric dehydrogenase was followed after the intraperitoneal injection of 0.01 mg. of cord factor, a statistically significant decrease of enzymatic activity was first noticed six hours after the injection, and the lowered enzymatic activity did not recover until seventy-two hours later (figure 1).

In Vitro Effect of Cord Factor on Succinic and α-Glycerophosphoric Dehydrogenase System of the Homogenate of Mouse Liver.

Normal mouse liver was homogenized in the presence of 0.25 M sucrose and mixed with 0.1 mg. of cord factor per ml. The mixture was kept at 0°C. and aliquots were taken at various intervals of time for the estimation of succinic and α-glycerophosphoric dehydrogenase activity. It

TABLE 3

EFFECT OF IN VITRO ADDITION OF CORD FACTOR ON SUCCINIC AND α-GLYCEROPHOSPHORIC DEHYDROGENASE ACTIVITY OF THE HOMOGENATE OF LIVER OF MICE

Time after the Addition of Cord Factor	γ of Formazan Reduced/mg.N/min.		
	Control	Propylene Glycol	Cord Factor
minutes		*10%*	*0.1 mg./ml.*
Succinic dehydrogenase			
0	6.8	7.0	6.9
30	6.6	7.3	6.8
60	6.3	6.8	7.2
120	7.3	7.8	7.9
180	6.1	6.7	7.1
α — Glycerophosphoric dehydrogenase			
0	3.6	4.5	3.5
30	3.2	4.5	3.3
60	3.2	2.8	3.3
120	4.4	2.8	3.3

Cord factor was dissolved in propylene glycol and added to the homogenate of liver to make final 0.1 mg. per ml. of cord factor and 10 per cent of propylene glycol. The mixture was kept at 0°C.

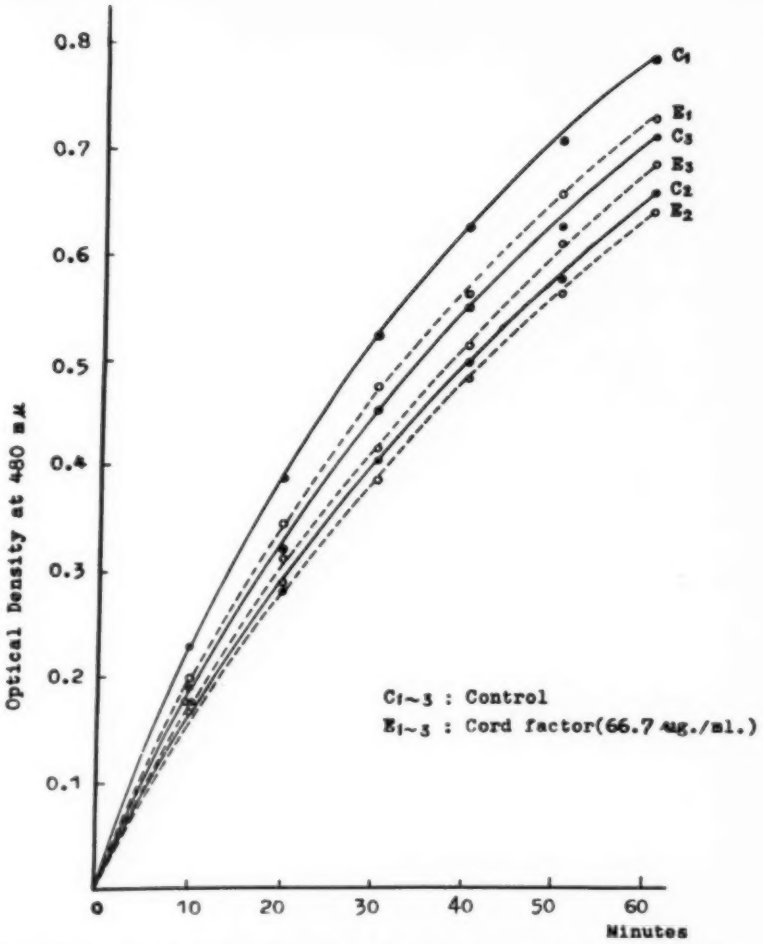

FIG. 2. Effect of *in vitro* addition of cord factor on the reduction of triphenyltetrazolium chloride (TTC) by the homogenate of normal liver with succinate as substrate. The reaction mixture consisted of: 0.5 ml. of 10 per cent homogenate (1.0 mg. N), 0.5 ml. of 0.2 M Na-succinate (100 μM), 1.0 ml. of 0.1 per cent TTC, and 1.0 ml. of 0.1 M phosphate buffer, pH 7.4.

may be seen in table 3 that the succinic and α-glycerophosphoric dehydrogenase activity of the homogenate was not affected by the presence of relatively high concentration *in vitro* of added cord factor during two to three hours.

As shown in figure 2, the addition of cord factor to the succinate-triphenyltetrazolium chloride system at 37°C. also did not exert any direct inhibition during sixty minutes on the reduction of the dye.

Reaction Site of Triphenyltetrazolium Chloride in the Succinic Dehydrogenase-Cytochrome System

It was attempted in the next experimental stage to ensure the reaction site of TTC in the long enzymic chain of succinic dehydrogenase-cytochrome system. The results summarized in table 4 show that the reduction of TTC by the mitochondria of bovine liver is completely inhibited by either antipirierin or cyanide. This

TABLE 4

EFFECT OF ANTIPIRICRIN AND CYANIDE ON THE SUCCINIC OXIDASE SYSTEM OF BOVINE LIVER MITOCHONDRIA

Hydrogence Acptor	Per Cent Inhibition	
	Antipiricrin	Potassium Cyanide (3×10^{-4} M)
	7γ per ml.	
TTC	100	80
O_2	92	—
$K_3Fe(CN)_6$	4	—

— = not tested.

TABLE 5

EFFECT OF CORD FACTOR ON SUCCINIC DEHYDROGENASE ACTIVITY OF MOUSE LIVER MEASURED WITH FERRICYANIDE AS HYDROGEN ACCEPTOR

Number of Mice	Decrease of Optical Density at 400 mμ per Mg. N per Minute ($\times 10^{-3}$)	
	Control	Cord-Factor-treated
(1)	134	90
(2)	110	99
(3)	111	117
(4)	114	88
Mean	117 ± 11	99 ± 10

Experimental conditions: liver homogenate; 0.4 mg. N, Na-succinate; 50 μM., KCN; 0.01 M., $K_3Fe(CN)_6$; 0.001 M.

Cord factor (0.1 mg./0.1 ml. olive oil) was injected 24 hours before the determination of enzyme activity.

indicates that TTC is reduced *via* the cytochrome system.

Effect of Cord Factor on Succinic Dehydrogenase, Succinic Oxidase, and Cytochrome Oxidase

As shown above, the reduction of triphenyltetrazolium chloride possibly involves cytochrome–cytochrome-oxidase system. Succinic dehydrogenase activity was compared between homogenates of liver of control and cord-factor–treated mice by measuring the reduction of ferricyanide and the oxygen consumption through methylene blue in the presence of cyanide.

Data summarized in tables 5 and 6 show that the injection of cord factor into mice lowers the activity of liver to reduce either ferricyanide or methylene blue with succinate as substrate. Succinic oxidase was also markedly decreased (table 6), but cytochrome oxidase was not affected by the injection of cord factor. It seems, therefore, that cord factor impairs in some way the enzymic activity of succinic dehydrogenase but not that of the cytochrome–cytochrome oxidase system.

Effect of Cord Factor on Succinic Dehydrogenase Activity of Homogenate and Mitochondrial Fraction of Mouse Liver

The liver was homogenized in the presence of 0.25 M sucrose, and the succinic dehydrogenase activity of the homogenate and of the mitochondrial fraction of the controls was compared with that of the cord-factor–treated mice. As shown in table 7, it was noted that cord factor caused a decrease of enzymic activity of homogenate without affecting that of the mitochondrial fraction of liver.

TABLE 6

EFFECT OF CORD FACTOR ON SUCCINIC DEHYDROGENASE, SUCCINIC OXIDASE, AND CYTOCHROME OXIDASE ACTIVITIES OF MOUSE LIVER

	μl. O_2 Uptake per Mg. N of Liver per Hour		
	Succinic Dehydrogenase	Succinic Oxidase	Cytochrome Oxidase
Control	642 ± 14.4	376 ± 11.8	316 ± 12.0
Cord-factor–treated	404 ± 22.0	184 ± 44.0	300 ± 24.0

Cord factor was injected 24 hours before the determination of enzyme activity.

Four animals in each group.

TABLE 7

EFFECT OF CORD FACTOR ON THE SUCCINIC DEHYDROGENASE ACTIVITY OF HOMOGENATE AND MITOCHONDRIAL FRACTION OF MOUSE LIVER

Enzyme Preparation	γ of Formazan Reduced per Mg. Dry Weight per 10 Minutes	
	Control	Cord-Factor-treated
Homogenate	3.7 ± 1.2	1.4 ± 0.77*
Mitochondrial fraction	1.7 ± 0.25	1.8 ± 0.76

Five animals in each group. Mitochondrial fraction was prepared as described in Materials and Methods.

* Indicates that the difference is significant ($\alpha = 0.01$).

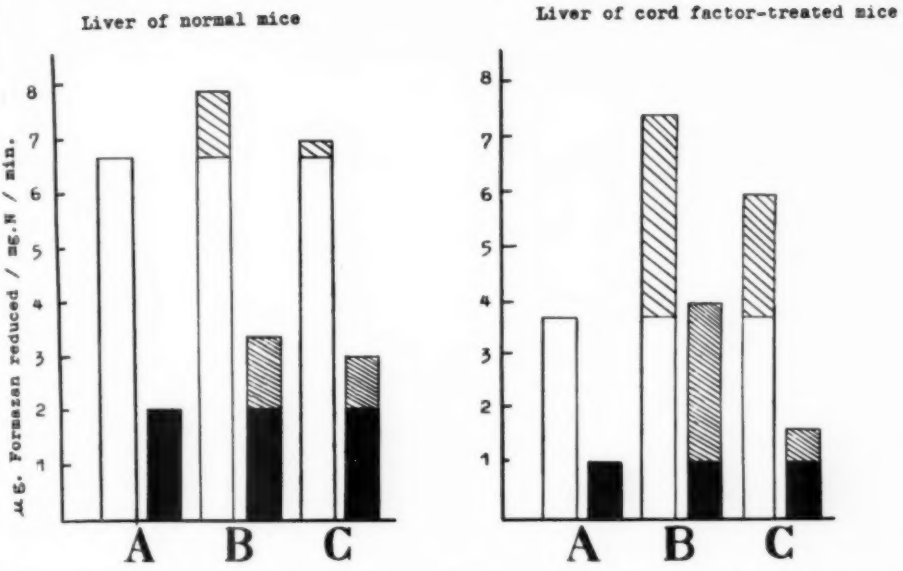

FIG. 3. Effect of sucrose extract of liver of normal and cord-factor–treated mice on succinic dehydrogenase and α-glycerophosphoric dehydrogenase activity of normal and cord-factor–treated mouse liver
A. Homogenized with 0.25 M sucrose
B. Homogenized with the sucrose extract of the liver of normal mice
C. Homogenized with the sucrose extract of the liver of cord-factor–treated mice.
Sucrose extract was prepared by centrifuging the 10 per cent homogenate of the liver of normal and cord-factor–treated mice, respectively, at 16,000 r.p.m. for 30 minutes. Figures represent the average of three tests.

□ : Succinic dehydrogenase
■ : α-Glycerophosphoric dehydrogenase
▨ ▩ : Activation of dehydrogenase activity by sucrose extract

Restoration of Decreased Enzymic Activity by Normal Liver Extract

It may be assumed from the above data that the decrease of succinic dehydrogenase activity by cord factor is related to the biochemical alteration in the soluble fraction of liver homogenates. In order to determine the nature of this alteration, livers of normal and cord-factor–treated mice were homogenized in the presence of 0.25 M sucrose, centrifuged at 16,000 r.p.m. for 30 minutes, and the supernatants were used to homogenize livers of normal and cord-factor–treated mice. The supernatants were free of succinic and α-glycerophosphoric dehydrogenase activity.

In the results summarized in figure 3, it may be seen that the supernatant of the homogenate of normal liver contains some factor which markedly activates the succinic dehydrogenase activity of the liver of both normal and cord-factor–treated mice. The supernatant of the homogenate of cord-factor–treated mice also exerts an activating effect, but at a lower rate. No inhibition of enzymic activity was noticed, however, by homogenizing the normal liver with the supernatant of cord-factor–treated mice. The enzymic activity of the liver of cord-factor–treated mice, when homogenized in the presence of normal supernatant, attains the same level as that of the normal liver homogenized with normal supernatant. Diphosphopyridine nucleotide, in the concentration of 0.1 mg. per ml., did not influence the succinic dehydrogenase activity of the liver of either normal or cord-factor–treated mice.

Discussion

There have been some reports on the inhibition of the succinic oxidase system of host tissues under the action of certain bacterial toxins or bacterial infection.

Pappenheimer (18, 19) obtained some experimental evidence to substantiate the assumption that the purified diphtherial toxin may block the biosynthesis of cytochrome *b* of intoxicated tissues and thus interfere with the succinic oxidase system.

MacFarlane (20), and MacFarlane and Datta (14) reported the inhibition of succinic oxidase activity of mitochondria by *Clostridium welchii* toxin. When mitochondrial suspensions were incubated with toxin under conditions suitable for the action of *Clostridium welchii* lecithinase, the succinic oxidase activity was greatly decreased in parallel with an increase in acid-soluble *P* through hydrolysis of mitochondrial phospholipid by toxin, but succinic dehydrogenase and cytochrome oxidase were less affected. The authors concluded that the effect was upon the intermediate factor transferring electrons from cytochrome *b* to cytochrome *c*. Nygaard and Sumner (21) reported a similar effect of crystalline lecithinase *A* (crotoxin) from snake venom on the succinic oxidase activity of rat liver mitochondria.

The decrease of the activity of the succinic dehydrogenase system of mouse liver by the injection of cord factor seems to be related to a mechanism different from those cited above.

Cord factor inhibits the succinic dehydrogenase of mouse liver estimated by the rate of reduction of ferricyanide and oxygen uptake *via* methylene blue in the presence of cyanide, while cytochrome oxidase is not affected. These data strongly suggest that cord factor attacks the component involved in the step near the reduction of substrate, and not in the cytochrome–cytochrome-oxidase system.

The effect of the cord factor was noted only *in vivo*. The activity of the succinic dehydrogenase system of liver homogenate was not affected by the *in vitro* addition of considerable amount of cord factor at 0° or 37°C. for a reasonable length of time. This indicates that cord factor does not directly inhibit the electron transfer from succinate to triphenyltetrazolium chloride. The possibility may be also excluded that cord factor acts as a detergent disintegrating the enzymic organization of the succinic oxidase system of mitochondria. It was therefore presumed that cord factor might interfere with the formation or biosynthesis of some component involved in the succinic dehydrogenase system.

Cord factor decreases the activity of the succinic dehydrogenase of liver homogenate, but not that of isolated mitochondria. This seems to indicate that the metabolic lesion caused by the injection of cord factor is not on the mitochondrial enzymes per se, but perhaps on the soluble factor which may influence in some way the activity of succinic dehydrogenase of mitochondria. This assumption was supported by the fact that the decreased enzymic activity by cord factor was restored by some component contained in the sucrose extract of normal liver. Although this restoration of enzymic activity was not specific to the decreased enzymic activity caused by the injection of cord factor, the quantitative decrease of this component by the injection of cord factor was proved in the cross experiment of the extract between normal and cord-factor–treated mice.

This substance is now under investigation. It is noted in experiments now in progress that the dialyzed extract heated at 100°C. for ten minutes retains the activating effect for the succinic dehydrogenase of the homogenate of the liver of both normal and cord-factor–treated mice.

Recently, Chaudhuri and Martin (22) and Martin and associates (23) called attention to the fact that infection with tubercle bacilli caused a significant decrease of the succinic dehydrogenase activity of the kidney of infected guinea pigs. These writers investigated the genesis of this lowering of enzymic activity and found that the decreased succinic dehydrogenase activity could be restored by a heat-labile and dialyzable factor which was obtained from normal guinea pig and bovine kidneys. This active agent was postulated to be desamino-coenzyme *A* (23, 24).

This work is very suggestive for the experiment described in the present paper. Investigations described herein also suggest that the possible metabolic lesion caused by the injection of cord factor is a quantitative decrease of a soluble factor or factors affecting the succinic dehydrogenase activity of mouse liver. It seems possible that the dialyzable factor reported by Martin and associates (23, 24) and the factor described in the present paper are the same. Further experiments on this point are now under investigation.

Summary

The mechanism of the inhibition of the succinic dehydrogenase system of mouse liver by cord factor was investigated.

The intraperitoneal injection of cord factor into mice caused the decrease of enzymic activity of succinic dehydrogenase and of certain diphosphopyridine nucleotide-linked (lactic, malic, and α-glycerophosphoric) dehydrogenase.

The minimal effective dose of cord factor to decrease the succinic dehydrogenase activity of mouse liver was 5 γ. The effect was noted only *in vivo*.

The decrease of succinic dehydrogenase activity by cord factor was confirmed both spectrophotometrically and manometrically. The activity of the succinic oxidase system was also decreased by the injection of cord factor, but the cytochrome oxidase was not affected.

The decreased enzymic activity (succinic and α-glycerophosphoric dehydrogenase) was restored by homogenizing the liver of cord-factor–treated mice with the sucrose extract of normal mouse liver.

The quantitative decrease of a soluble factor affecting the activity of the succinic dehydrogenase system of mouse liver was assumed to be the metabolic lesion caused by the injection of cord factor.

Sumario

El Mecanismo de la Toxicidad del Factor Cordón

Esta investigación versa sobre el mecanismo de la inhibición del sistema de la dehidrogenasa succínica del hígado de ratón por el factor cordón.

La inyección intraperitoneal del factor cordón en los ratones hizo disminuir la actividad enzimática de la dehidrogenasa succínica y de cierta dehidrogenasa difosfopiridínica combinada con nucleótidos (láctico, málico y o[(-glicerofosfórico])).

La dosis eficaz mínima del factor cordón para rebajar la actividad de la dehidrogenasa succínica del hígado de ratón fué de 5γ. No se notó el efecto más que *in vivo*.

La disminución de la actividad de la dehidrogenasa succínica ocasionada por el factor cordón quedó confirmada tanto espectrofotométrica como manométricamente. También hizo disminuir la actividad del sistema de la oxidasa succínica la inyección del factor cordón, pero no se afectó la oxidasa citocrómica.

Se restableció la disminuída actividad enzimática (dehidrogenasa succínica y o[-glicerofosfórica]) homogeneizando el hígado de los ratones tratados con el factor cordón con el extracto sacarosado del hígado de ratón normal.

Se dió por sentado que la disminución cuantitativa de un factor soluble que afectaba la actividad del sistema de la dehidrogenasa succínica del hígado murino representaba la lesión metabólica ocasionada por la inyección del factor cordón.

Resume

Le mécanisme de la toxicité du "cord factor"

Le mécanisme de l'inhibition du système déhydrogénase succinique du foie de souris par le "cord factor" a été étudié.

L'injection intrapéritonéale du "cord factor" à la souris a entraîné une réduction de l'activité enzymatique de la déhydrogénase succinique et d'une déhydrogénase diphosphopyridine liée à un nucléotide(lactique, malique et α-glycérophosphorique).

La dose minimale de "cord factor" efficace pour réduire l'activité déhydrogénase succinique du foie de souris a été de 5γ. Cet effet n'a été observé que *in vivo*.

La réduction de l'activité déhydrogénase succinique par le "cord factor" a été confirmée à la fois par spectrophotométrie et par manométrie. L'activité du système oxydase succinique a été également déprimée par l'injection du "cord factor," mais l'oxydase cytochrome n'a pas été affectée.

Cette activité enzymatique réduite (déhydrogénase succinique et alpha-glycérophosphorique) a été restaurée par homogénéisation du foie de souris traitées par le "cord factor" avec le saccharose extrait de foie de souris normales.

L'auteur attribue la réduction quantitative d'un facteur soluble influençant l'activité du système déhydrogénase succinique de foie de souris à une lésion métabolique causée par l'injection du "cord factor."

Acknowledgment

The writers' grateful thanks are due to Dr. Hubert Bloch for supplying the purified sample of cord factor for this study and for kindly reviewing the manuscript.

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Case Reports

PULMONARY ALVEOLAR PROTEINOSIS[1]

A Case Report with Unusual Clinical and Laboratory Manifestations

FRANCIS B. LANDIS, HAROLD D. ROSE, AND RICHARD O. STERNLIEB

(Received for publication April 3, 1959)

INTRODUCTION

In 1958, Rosen, Castleman, and Liebow (1) called attention to the unusual pulmonary disorder which they called pulmonary alveolar proteinosis. Their report was based on pathologic material gathered from 27 cases observed since 1953. The distinguishing pathologic features include: a filling of alveolar spaces with a proteinaceous material, PAS positive, and rich in lipids; and the presence of granules and variable laminated bodies. The similarity of symptomatology, roentgenographic pattern, clinical course, and histologic picture in the 27 cases served to establish this disorder as a distinct entity. The etiology remains obscure.

The present case, probably the first detected in Wisconsin, is being reported because of certain interesting features which may contribute to the clinical picture of this little known disorder.

CASE REPORT

R. S., a 48-year-old white, male, filling-station operator, who lived in northern Wisconsin, was admitted to the Medical Service, Veterans Administration Hospital, Wood, Wisconsin, on July 25, 1958, with acute thrombophlebitis of the right leg. He stated that he had been well until the summer of 1957 when he developed an acute illness, manifested by chills, fever, and chest pain. A clinical diagnosis of pneumonia was made, and he received one injection of penicillin, followed by a three-day course of a broad-spectrum antimicrobial. A chest roentgenogram was not obtained. The acute phase of the patient's illness subsided in a few days but he did not recover completely. During subsequent months, he noticed morning cough, productive of small amounts of yellowish sputum, brief hemoptysis on two occasions, easy fatigability, mild exertional dyspnea, anorexia, and a gradual weight loss of 40 pounds.

In December, 1957, the patient developed pain in the left calf and swelling of the dorsum of the left foot, subsiding after four days. Signs of thrombophlebitis reappeared in May, 1958, with pain and swelling of his right thigh, again subsiding after a week of symptomatic treatment. He experienced a third attack in July, 1958, involving his right calf, following which he was referred to the Veterans Administration Hospital, Wood, Wisconsin. None of the attacks of phlebitis were accompanied by hemoptysis or chest pain.

Past history: Past history disclosed the usual childhood illnesses, including scarlet fever without sequelae. The patient's only previous hospitalization was in 1946 for a head injury sustained in an automobile accident. No chest roentgenogram was obtained after 1946 until his present illness. In May, 1958, during an episode of thrombophlebitis, albumin was detected in the patient's urine. He denied any allergic disorder, and his family history was noncontributory.

Occupation and habits: For the past seven years the patient had operated a small filling station, pumping gas and performing minor automobile repairs. He denied excessive exposure to exhaust fumes or sprays. For the past ten years he had also worked part time as a bartender. He admitted an excessive alcoholic intake with poor dietary habits, particularly after the onset of his present illness. His smoking habit included one to two packs of cigarettes daily for thirty years.

Physical examination: Physical examination disclosed a well-developed, fairly well-nourished, white male, not acutely ill, and in no respiratory distress. Temperature, pulse, and respiration were normal; his weight was 154 pounds; blood pressure, 110 systolic and 64 diastolic in mm. of mercury. Pertinent findings included moist rales audible in both lung bases and in the right axilla. The liver was palpable 5 cm. below the right costal margin, and was firm, smooth, and nontender. Dilatation and tortuosity were noted in the veins of the right lower leg, with slight tenderness and increased warmth of the right calf, which measured one inch larger than the left. Erythema was absent; Homan's sign was equivocal. Clubbing of fingers and toes was not present.

Laboratory data: The hemoglobin was 15.3 gm.; the leukocyte count was 13,100 per cu. mm. with a slight shift to the left. Subsequent determinations were repeatedly normal. The erythrocyte sedimentation rate varied from 47 to 87 mm. in one

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hour (Westergren). Urinalysis disclosed proteinuria primarily during the early phase of illness. Urine culture was sterile. The maximal urine concentration on the Fishberg test was 1.017; nonprotein nitrogen ranged from 31 to 44 mg. per 100 ml. The phenolsulfonphthalein test on August 22, 1958, revealed that 18.9 per cent of dye was excreted in fifteen minutes and 38.3 per cent in one hour. A repeat phenolsulfonphthalein test on January 9, 1959, revealed 15 per cent excretion in fifteen minutes and 34 per cent in one hour (lower limits of normal are 20 and 40 per cent, respectively). The bromsulfalein, serum bilirubin, prothrombin time, and alkaline phosphatase tests were repeatedly normal.

Thymol turbidity (5×) varied from 13.0 to 47.0 units; zinc sulfate turbidity (7×) varied from 13.6 to 26.2 units. On two occasions, when significant elevations of thymol turbidity (47.0 units) and zinc sulfate (26.2 units) were reported, the laboratory technician commented on the hyperlipemic appearance of the patient's serum.

A chronologic list of pertinent laboratory data, including proteinuria, blood proteins, electrophoretic pattern, cholesterol, and serum lipids, is shown in table 1. Unfortunately, the 24-hour quantitative urine protein determination, total blood lipid, lipid phosphorus, and electrophoresis were not obtained until the later stage of the patient's illness, when clinical improvement was already apparent. Fungal skin tests for blastomycosis, histoplasmosis, and coccidioidomycosis were negative; a tuberculin test (second-strength PPD) was positive. Sputum cultures (3×) and bronchial washings were negative for tubercle bacilli. Candida was recovered on one culture of sputum for fungi (3×). Routine sputum examinations for predominating organisms (7×) disclosed pneumococci twice; normal flora, five times. Cold and febrile agglutinins were negative.

Ventilatory function tests, August 4, 1948, were as follows: vital capacity, 3.2 liters (predicted normal 4.3 liters); timed vital capacity, 80 per cent exhaled in one second, 91 per cent exhaled in three seconds. Maximal breathing capacity was 134 liters (predicted 110 liters). Following administration of a bronchodilator drug, there was no significant change in the above figures. Ventilatory tests were repeated on November 18, 1958, and on January 5, 1959; vital capacity increased to 3.5 liters on the latter date. Arterial blood gas analysis on September 11, 1958, revealed resting arterial oxygen saturation 96.4 per cent; following exercise, 98.7 per cent; and with inhalation of 100 per cent oxygen, 100 per cent saturation. Carbon dioxide tension at rest was 43 mm. of mercury; with exercise, 41 mm. of mercury; and with inhalation of 100 per cent oxygen, 38.5 mm. of mercury. Lung volume studies by the helium-dilution method on November 10, 1958, disclosed: vital capacity, 2.6 liters (predicted 4.3 liters); residual volume, 1.17 liters (predicted 1.31 liters); and total lung capacity, 3.78 liters (predicted 5.6 liters). Residual air was 31 per cent (normal below 35 per cent). Pulmonary function tests were interpreted as

TABLE 1

CHRONOLOGIC LISTING OF SIGNIFICANT LABORATORY DATA

	Proteinuria		Serum Proteins			Serum Proteins (Paper Electrophoresis)					Serum Cholesterol		Total Blood Lipid	Lipid Phosphorus
	Routine Urinalysis	24-Hour Quantitative	Total	Albumin	Globulin	Albumin	Alpha$_1$	Alpha$_2$	Beta	Gamma	Total	Free		
Normal	Negative	Less than 0.1 gm.	6.0–8.0 gm. per 100 ml.	3.7–4.6	2.25–3.5	4.16	0.32	0.62	0.58	1.37	170–290 mg. per 100 ml.	24–32 % of total	400–800 mg. per 100 ml.	6.1–14.5 mg. per 100 ml.
Date:														
7/28/58	3+		6.44	2.38	4.06									
8/20/58	1+		7.97	3.00	4.97						312	125		
9/2/58	4+													
9/20/58			8.37*	3.37	5.00						334	144		
10/14/58			7.43*	2.38	5.05						427*	206		
10/20/58	3+													
11/12/58	Trace										263	85		
11/19/58						3.23	0.36	0.61	0.92	1.73				
12/17/58	4+		7.83	4.00	3.83						307	104		
1/6/59	Negative	0.51	7.00	3.68	3.32	3.00	0.54	0.78	0.96	1.72	158	69	975	6.7
1/15/59	Negative	Negative	7.77	3.88	3.89									
1/16/59		Negative												
1/26/59	Negative													

* Markedly hyperlipemic serum reported by laboratory technician

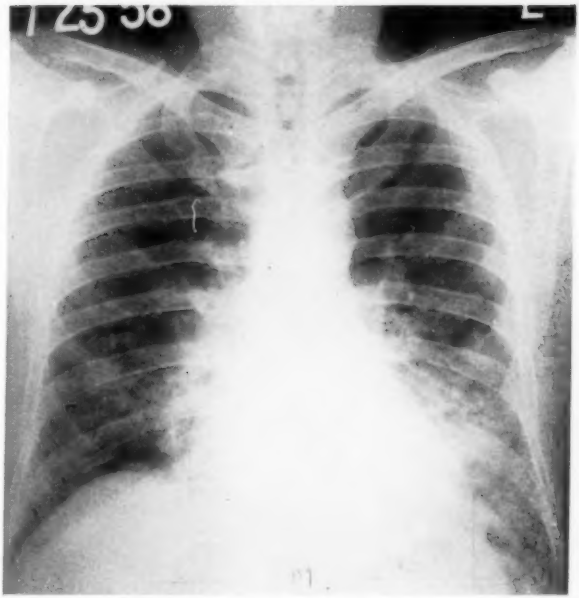

FIG. 1. Roentgenogram of July 25, 1958, taken on admission, reveals bilateral feathery and soft infiltration in both lower lung fields, more extensive on the left.

showing slight restrictive ventilatory impairment due to pulmonary infiltration.

Bronchoscopy on September 23, 1958, demonstrated a normal tracheoendobronchial tree. Electrocardiograms, repeated on three occasions, were normal.

The admission chest roentgenogram on July 25, 1958 (figure 1) revealed bilateral feathery and soft infiltrations in both lower lung fields, more extensive on the left. Progress films of August 25, 1958, and September 2, 1958, demonstrated a transient spread of disease in the lower third of the right lung with partial clearing already apparent on the film of September 10, 1958. The probability of a superimposed bacterial pneumonitis was considered unlikely, since there was neither exacerbation of symptoms nor fever during the interval. The maximal pulmonary involvement was noted on the roentgenogram of September 2, 1958. Thereafter, no change was detected until December, 1958, when slight bilateral clearing was apparent. The most recent film, on January 28, 1959, shows light scattered, feathery infiltration in the right lung and heavier confluent infiltration at the left base. The roentgenographic pattern in this patient did not resemble the typical perihilar butterfly distribution simulating pulmonary edema, described in Rosen's series. Gastrointestinal series, gall bladder series, barium enema, and excretory urogram were normal.

Clinical course and treatment: Following admission, the differential diagnosis included cardiac failure with congestive pulmonary changes, pneumonia of bacterial or viral type, and pulmonary infarction, secondary to recurrent phlebothrombosis. Also, cryptic malignancy was suspected because of unexplained weight loss and recurrent thrombophlebitis. The patient was started on digitalis and anticoagulants, given diuretics, and maintained on a low-sodium cardiac diet. His venous pressure and circulation times were normal, and the cardiac regimen did not appreciably alter his clinical course or roentgenographic findings. Thus, cardiac failure was excluded. The signs of thrombophlebitis subsided and anticoagulants were discontinued on September 4, 1958. The distribution and appearance of the bilateral pulmonary lesions, with failure to clear on serial films, did not seem consistent with pulmonary infarction. The patient received courses of penicillin, chloramphenicol, and novobiocin with no appreciable influence on the pulmonary picture; and except for occasional low-grade fever, he remained afebrile during his hospital course, making a diagnosis of pneumonia untenable.

In late September, 1958, the patient was presented to the Medical Chest Conference, at which the diagnosis of pulmonary alveolar proteinosis was suggested. Scalene lymph node and lung biopsies were recommended. Right cervico-

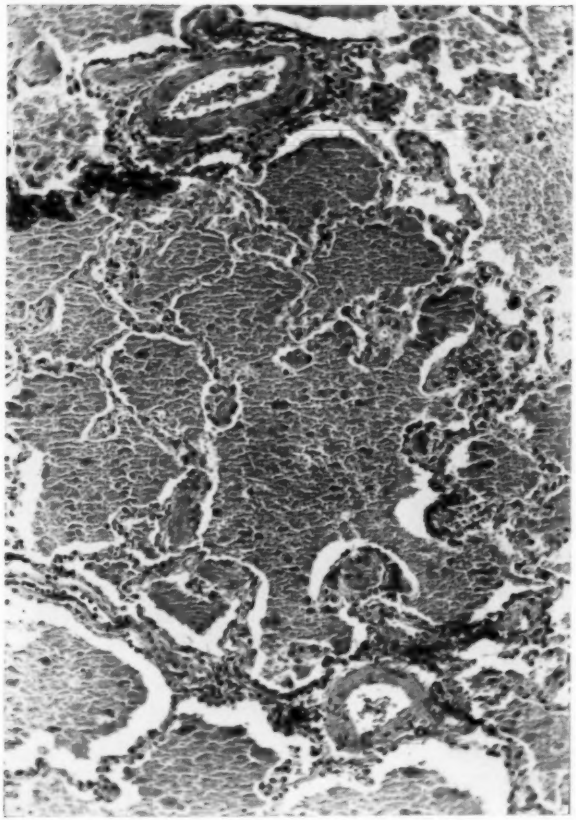

FIG. 2. Lung biopsy. The alveoli are distended by amorphous, eosinophilic, proteinoid material which stains with variable intensity to the PAS technique. (Hematoxylin-eosin stain, × 130)

mediastinal lymph node biopsy on October 1, 1958, was normal. On October 21, 1958, a wedge biopsy was obtained from the medial lingular segment of the left upper lobe, revealing the distinctive pathologic features of pulmonary alveolar proteinosis.

A sterile, fresh portion of the biopsy material was sent directly to the bacteriology laboratory. The technician who examined the specimen stated that a purulent-appearing fluid was easily expressed from the cut surface and, somewhat to his surprise, the Gram stain of this fluid failed to reveal either leukocytes or bacteria. Special stains revealed neither fungi nor cysts of pneumocystis carinii. Cultures for bacteria, fungi, and acid-fast bacilli were negative. The fixed specimen did not appear unusual. The cut surface was described as moderately firm, grayish-tan, with a finely granular texture. The microscopic sections (figure 2) were reported as follows: throughout the parenchyma the alveoli were distended by granular, eosinophilic, proteinoid material containing occasional elongated, narrow, crystalline clefts. In scattered areas, the proteinoid material within the alveoli was condensed into fairly dense, eosinophilic concretions. The proteinoid material stained with variable intensity by the PAS technique. The areas of condensation of proteinoid material were particularly positive, whereas many of the looser granular areas were negative to this special stain.

The lung biopsy slides were reviewed by Dr. S. H. Rosen, Armed Forces Institute of Pathology, who concurred in the diagnosis of pulmonary alveolar proteinosis. He added that:

> There are other microscopic findings that suggest the possibility of a superimposed infection, namely the focal edema of the pleura, and the presence of a few small foci of what appear to be organizing fibrin on

the pleura; and the presence in one lobule of a few alveoli containing necrotic cells, among which can be seen polys and an occasional eosinophil.

In November, 1958, the patient was treated with buccal varidase and superinone (Alevaire®)-vaponephrine by nebulizer four times daily. He was almost symptom free at this time, and no definite clinical or roentgenographic improvement was noted. He was discharged on November 20, 1958, with recommendation to continue isoproterenol (Isuprel®)-superinone by nebulizer, and buccal varidase.

When re-examined on December 16, 1958, he had gained 13 pounds and felt well except for mild exertional dyspnea. A chest roentgenogram on December 16, 1958, revealed slight clearing bilaterally.

On January 4, 1959, the patient was readmitted for further study, and renal and liver biopsies were obtained. Needle biopsy of the kidney on January 7, 1959, revealed the following microscopic appearance: The glomerular tufts were normal; many of the capsular spaces contained an albuminoid, homogeneous to granular eosinophilic precipitate. The tubular epithelium was well preserved. Hyalin casts were occasionally evident and stained positively by the PAS technique. Occasional loops of Henle were dilated with partial flattening of their lining epithelial cells. These changes were not considered diagnostic of any specific entity. Intercostal needle biopsy of the liver on January 9, 1959, was normal. A chest roentgenogram, January, 1959, showed no significant change.

The patient was discharged and presently has minimal symptoms and works daily in his gasoline filling station. He will be followed at intervals.

Discussion

Pulmonary alveolar proteinosis, as described by Rosen, occurs chiefly in adults and is characterized by insidious onset, slow progression, cough, increasing fatigue, chest pain, dyspnea, and weight loss. The first manifestation is often a febrile illness, but fever is usually slight or absent during the prolonged clinical course. The typically roentgenographic picture consists of a bilateral perihilar butterfly infiltration, simulating pulmonary edema. The pulmonary lesions detected roentgenographically may persist for months or years; may clear, remain stationary, or progress. The etiology is unknown, but since the lung appears to be the primary site of involvement, noxious inhalants have been suspected.

Since Rosen's original paper, few reports have appeared describing proteinosis. Payseur and associates (2) reviewed the ninth case in Rosen's series at greater length and pointed out that this patient originally had active pulmonary tuberculosis which had responded to antituberculous drugs, and returned three years later with pulmonary alveolar proteinosis. Single case reports from Veterans Administration hospitals, Dearborn, Michigan, and Livermore, California, have appeared in the October, 1958, Veterans Administration Quarterly Report of Tuberculosis (3). Since this recently described disorder poses a diagnostic challenge to physicians, it is probable that other cases will be discovered in increasing numbers; also, retrospective analysis of pathologic material from undiagnosed pulmonary cases of former years may, in some instances, demonstrate the distinctive pathologic features of proteinosis.

The present case presents several interesting features. Thrombophlebitis, not previously reported in association with proteinosis, recurred on three occasions. The occurrence of thrombophlebitis, pulmonary symptoms, and infiltrates in the lower lung fields strongly suggested pulmonary infarction and probably delayed establishment of the correct diagnosis. The significance of thrombophlebitis occurring in a patient with proteinosis is unknown; it may be coincidence.

There are also unexplained laboratory findings, including elevated serum lipid and total cholesterol, hyperglobulinemia, and proteinuria. Analysis of the laboratory tests in table 1 indicates that the greatest deviation from normal occurred during the early months of hospitalization when roentgenographic involvement was also most marked. It is unfortunate that liver and renal biopsies were not obtained at that time, for pathologic changes may have been present which would materially aid in answering the following questions: (*1*) Was the enlarged liver secondary to fatty infiltration in an alcoholic, poorly nourished patient? (*2*) Do hepatic and renal changes occur in proteinosis? (*3*) Was a renal disorder, unrelated to proteinosis, responsible for proteinuria, or did the patient actually have an incomplete nephrotic syndrome, possibly related to proteinosis?

If similar findings are observed by others studying this most interesting disorder, it is hoped that careful coordination of laboratory data and biopsies will provide answers.

Summary

The case of a 48-year-old white male with pulmonary alveolar proteinosis proved by lung biopsy is reviewed. Features of interest include recurrent thrombophlebitis, hepatomegaly, proteinuria, hyperglobulinemia, and elevation of blood cholesterol and total lipids. Significant clinical and slight roentgenographic improvement occurred.

Sumario

Proteinosis Alvéolpulmonar: Presentación de un Caso con Extrañas Manifestaciones Clínicas y de Laboratorio

Repásase el caso de un sujeto blanco de 48 anos de edad, que tenía proteinosis de los alvéolos pulmonares, comprobada por la biopsia del pulmón. Las características interesantes comprenden: tromboflebitis recurrente, hepatomegalia, albuminuria, hiperglobulinemia y elevación de la colesterina sanguínea y de los lípidos totales. Hubo notable mejoría clínica y ligera radiográfica.

Resume

Protéinose alvéolaire pulmonaire: Rapport sur un cas avec manifestations cliniques et expérimentales inhabituelles

Compte-rendu d'un cas concernant un homme de race blanche âgé de 48 ans présentant une protéinose alvéolaire pulmonaire confirmée par biopsie du poumon. Les traits caractéristiques étaient les suivants: thrombophlébite récurrente, hépatomégalie, protéinurie, hyperglobulinémie et élévation du cholestérol sanguin et des lipides totaux. Il s'est produit une amélioration qui était notable du point de vue clinique et lègére du point de vue radiologique.

Acknowledgment

Grateful acknowledgment is extended to Dr. Russell Maynard, Chief of Pathology, Veterans Administration Hospital, Wood, Wisconsin, for his concise description of the biopsy specimens and for his confirmation of the diagnosis.

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(1) Rosen, S. H., Castleman, B., and Liebow, A. A.: Pulmonary alveolar proteinosis, New England J. Med., 1958, *258*, 1123.

(2) Payseur, C. R., Konwaler, B. E., and Hyde, L.: Pulmonary alveolar proteinosis: A progressive, diffuse, fatal, pulmonary disease, Am. Rev. Tuberc., 1958, *78*, 906.

(3) Quarterly Progress Report of the Veterans Administration-Armed Forces Study on the Chemotherapy of Tuberculosis: Case reports from Dearborn VA and Livermore VA, October, 1958, XIII.

Editorial

Standardization and Stability of Purified Tuberculin

A recent issue of the *Bulletin of the World Health Organization*[1] contains a collection of five papers on purified tuberculin (PPD). The papers have been prepared by the WHO Tuberculosis Research Office and the Statens Seruminstitut, Copenhagen. In addition to new information on the stabilization and storage of diluted tuberculin, the papers are also concerned with a new, very large batch of purified tuberculin (designated RT 23) recently produced in the Statens Seruminstitut, Copenhagen, at the request of UNICEF.

The first three papers are reports on experiments on the stability of PPD when diluted as for use in intradermal testing, that is, in the ratio 1:1,000,000 or thereabouts. The experiments were carried out by skin testing BCG-vaccinated guinea pigs and BCG-vaccinated schoolchildren. Some of these experiments have been reported in preliminary form in two Notes in this journal.[2, 3]

The subject of the first of the present papers[4] is long-term storage, and demonstrates that, in the main, tuberculin dilutions are surprisingly stable. Even after six months' storage at 37°C., only a slight deterioration in tuberculin potency (about 25 per cent) is observed, and no deterioration is found after storage at 2 to 4°C. for as long as eighteen months. It is concluded that tuberculin in diluted form, ready for use, may be distributed in simpler and less expensive ways than have hitherto often been used.

The data from these studies of storage do, however, show unpredictable variations in potency between dilutions prepared in the same way from the same stock solution, and even differences in potency between ampoules filled with tuberculin dilution from the same container. These variations, which are independent of time, are sometimes much larger than the systematic deterioration with time, so that it is difficult to measure the latter precisely. These unsystematic variations are further examined in the second paper,[5] together with another manifestation of instability observed by the writers, namely, that a tuberculin dilution kept in a bottle or ampoule which is only partially filled loses most of its potency (per unit volume) compared with a similar dilution kept in a bottle which is completely filled. Evidence is offered that these phenomena are solely due to adsorption to the glass walls of bottles and ampoules of more than half of the diluted tuberculin —sometimes much more than half—so that it is not surprising if the small amount of unadsorbed tuberculin varies from ampoule to ampoule.

The third of the papers[6] gives further information on the dependence of the adsorption on the diluent used. That tuberculin diluted in saline is weaker than tuberculin diluted in buffer has been known for a long time, but this effect is now seen to be due to increased adsorption. The adsorption may be further inhibited by the use of other additives besides buffer salts; proteins such as blood albumin or gelatin reduce the adsorption considerably but, of all of the additives tested so far, the nonionic detergent "Tween® 80" gives the strongest and most constant stability. The very striking increase in potency when Tween 80 is added might suggest that this agent not only prevents adsorption of tuberculin to glass, but also has an enhancing effect on the tuberculin reaction *in vivo*; however, the writers have examined this possibility and found some evidence against it.

The powerful stabilization obtained with Tween 80 suggests that this additive might be used to advantage in the field to avoid erratic variations in potency. However, this would be inadvisable if Tween 80 had any sensitizing effect, alone or in conjunction with tuberculin. Experiments in guinea pigs and rabbits were carried out to study this possibility and seemed to exclude entirely the likelihood of sensitization with Tween 80 occurring under the ordinary conditions of tuberculin testing. The writers therefore suggest that Tween 80 (in a concentration of 0.05 per cent) be used as a matter of routine in the future as additive to the tuberculin diluent so as to prevent unpredictable variations in the potency of the dilutions.

The last two papers give information on the new batch of tuberculin (RT 23) referred to above. The total of this batch is 670 gm. (a very large amount indeed), and 500 gm. of this is the property of UNICEF.

The main advantage of having a very large

[1] Bull. World Health Organ., 1959, *19*, 759–951.

[2] Guld, J., *et al.*: Am. Rev. Tuberc., 1955, *72*, 126.

[3] Magnus, K., *et al.*: Am. Rev. Tuberc., 1956, *74*, 297.

[4] Magnus, K. *et al.*: Bull. World Health Organ., 1958, *19*, 765.

[5] Waaler, H., *et al.*: Bull. World Health Organ., 1958, *19*, 783.

[6] Magnusson, M., *et al.*: Bull. World Health Organ., 1958, *19*, 799.

batch is that it obviates the necessity for frequent standardization of new batches of tuberculin; it also makes it possible for the users to get thoroughly acquainted with the particular properties of the batch and to adjust dosage, et cetera, accordingly. In epidemiologic investigations to be continued over a number of years, it is an advantage if the same tuberculin product can be used throughout.

The preparation of this batch is described in the fourth paper.[7] The tuberculin was made according to Dr. Florence Seibert's prescription from 1934 (with minor modifications), as were previous batches from the Statens Seruminstitut. Cultures of tubercle bacilli on a synthetic medium were subjected to heat treatment, sterile filtration, ultrafiltration, and precipitation with trichloracetic acid, and the precipitate was dehydrated with ether. Seven different strains of tubercle bacilli of human origin were used and several sub-batches were made from each. After the sub-batches had been found to be fairly uniform in potency, by testing in guinea pigs, they were pooled and thoroughly mixed. The new tuberculin was standardized in human populations in the Netherlands, Nigeria, Mauritius, and Denmark. It was also standardized in guinea pigs sensitized in several ways. The standardization included not only a comparison with the International Standard for the purified protein derivative of mammalian tuberculin (PPD-S), but also with the earlier products from the Statens Seruminstitut, RT 19-20-21 and RT 22. For RT 23 both buffer diluent with Tween 80 and buffer diluent without Tween 80 were used. For the other tuberculins only buffer diluent without Tween 80 was used.

The analysis of the results, together with a complete documentation of the experimental data, is given in the fifth paper.[8] This analysis gave very definite evidence of "qualitative" differences between the tuberculin products, so that no single, absolute standardization is possible. For example, the potency ratio of the RT tuberculins and the International Standard is not the same in BCG-vaccinated and in nonvaccinated, tuberculin-sensitive persons. There is even a peculiar, qualitative difference between RT 23 with and without Tween 80 in that, for the same reaction size, reactions to tuberculin with Tween 80 are softer and less often show bullae or vesicles. The results in guinea pigs are also different from the results in humans. In the light of the results in naturally infected humans, in terms of reaction size, it is suggested that the following doses be considered equipotent for practical purposes:

0.000084 mg.	(including 0.000024 mg. of buffer salts) of the International Standard (3 TU)
0.0001 mg.	of RT 19-20-21 ("5 TU")
0.00009 mg.	of RT 22 ("7 TU")
0.00006 mg.	of RT 23 without Tween 80
0.00002 mg.	of RT 23 with Tween 80.

To keep in agreement with the terminology of the existing definition of the International Tuberculin Unit, it seems necessary to define one unit of RT 23 in terms of dry weight and irrespective of the diluent used. As 0.00006 mg. of RT 23 (diluted without Tween) corresponds to 3 TU of the International Standard (diluted without Tween), one unit of RT 23 thus becomes 0.00002 mg. of the dry substance. This means that the dilution of RT 23 with Tween 80 which may substitute "5 TU" of RT 19-20-21 must be labeled "one tuberculin unit." This is regretted by the writers, as it will no doubt cause some confusion.

If 0.00002 mg. of RT 23 stabilized with Tween 80 is accepted as the future standard test, there is (neglecting losses) a total of 33,000,000,000 doses of 0.00002 mg. each in the whole batch. Even if this is not enough to cover world requirements until the complete eradication of tuberculosis has been effected, it may last for a great many years, very likely until after it has been outmoded by new types of tuberculin, especially tuberculins with selected properties of specificity.

J. GULD
Copenhagen, Denmark

[7] MAGNUSSON, M., AND BENTZON, M. W.: Bull. World Health Organ., 1958, *19*, 829.

[8] GULD, J., *et al.*: Bull World Health Organ., 1958, *19*, 845.

Notes

STUDIES OF THE CATALASE ACTIVITY OF MYCOBACTERIUM TUBERCULOSIS STRAIN H37Rv

The majority of the methods thus far used in investigations of the catalase activity of tubercle bacilli are semi-quantitative,[1-3] and they do not permit determination of the specific catalase activity at zero time.

For the purpose of these investigations, a modification was made of the standard iodometric method[4] of the determination of catalase activity. The procedure is as follows:

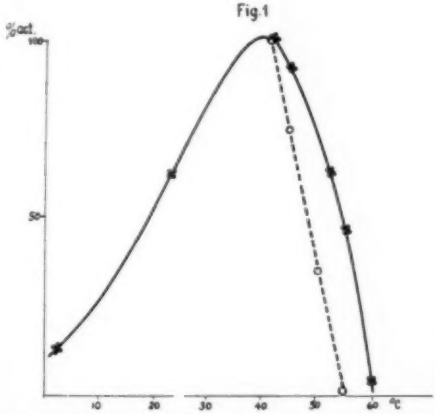

FIG. 1. The effect of temperature on the catalase activity of *Mycobacterium tuberculosis*. The aqueous suspension of *M. tuberculosis* strain H37Rv was prepared from a five-week-old culture on Sauton medium, washed three times with distilled water, and ground with glass beads. For the determination of activity (marked by crosses on the curve), 1 ml. of the suspension at room temperature was taken and added to 25 ml. of phosphate buffer previously brought to a desired temperature in a water bath. The samples were taken at zero, three, six, and nine minutes, and the k_0 was calculated.

Stability: The dotted curve was investigated by keeping the suspensions for ten minutes at the indicated temperature, and then immediately transferring them to the temperature of 37°C. for activity determinations, and the k_0 was calculated.

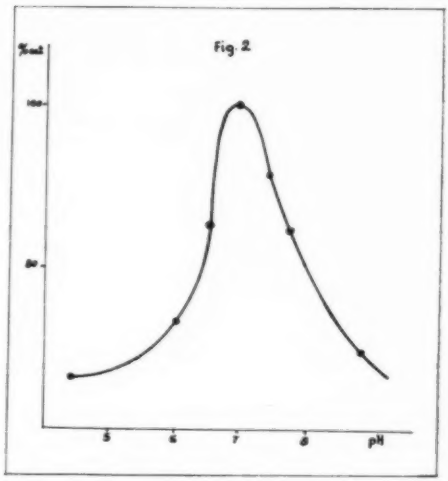

FIG. 2. The influence of pH on the catalase activity of *Mycobacterium tuberculosis*. The suspension was prepared from an eight-week-old culture, as in figure 1. One milliliter of aqueous suspension of the bacilli was added to the M/15 phosphate buffer of the desired pH containing H_2O_2. The flasks containing buffer were placed in a water bath at 37°C., and the k_0 was calculated.

One milliliter of the investigated suspension of bacilli (density 40 to 400 mg. moist weight per ml.) is put into a 50-ml. Erlenmeyer flask containing 25 ml. of 0.01 NH_2O_2 in M/15 phosphate buffer at pH 7, and placed in a water bath at 37°C. Immediately after mixing, a 5-ml. sample is taken by means of a pipette and blown into a flask containing 5 ml. of 20 per cent trichloracetic acid at the same time that a stop watch is started. Further samples are taken at intervals of three, six, and nine minutes. The first sample is denoted as zero. After mixing with trichloracetic acid, the samples are put aside for two to three minutes in order to precipitate the bacilli, are then filtered

[1] MIDDLEBROOK, G.: Am. Rev. Tuberc., 1954, *69*, 471.

[2] PEIZER, L. R., AND WIDELOCK, D. L.: Am. Rev. Tuberc., 1955, *71*, 305.

[3] SCHWEIGER, O., *et al.*: Am. Rev. Tuberc., 1958, *77*, 146.

[4] COLOWICK, S., AND KAPLAN, N.: In *Methods in Enzymology*, Vol. II, Academic Press, Inc., New York, 1955, p. 780.

on the paper (highly active suspensions of low density do not need to be filtered). Seven milliliters of the filtrate are taken, and 1 ml. of a 5 per cent solution of potassium iodide and 0.5 ml. of saturated molybdic acid solution are added. Three minutes after mixing, the samples are titrated with 0.005 N sodium thiosulfate in the presence of a few drops of starch. The velocity constant (k_0) of the reaction is determined by extrapolation of the value k obtained in three, six, and nine minutes as against the value zero. The values k are calculated from the formula:

$$k = \frac{1}{t} \log \frac{A}{A\text{-}x}$$

in which:

t = time in minutes
A = the amount of $Na_2S_2O_3$ in milliliters used for the sample zero
$A\text{-}x$ = the amount of $Na_2S_2O_3$ in milliliters used for the three-, six-, and nine-minute samples.

k_0 divided by the quantity of milligrams of nitrogen contained in the 1-ml. sample gives the specific catalase activity of the suspension used. The error of the method is within 3 per cent.

Some of the data are given in figures 1 and 2. In figure 1 it is shown that the catalase activity of the tubercle bacilli increases with the temperature from 0°C. to 45°C. Catalase is inactivated completely in the stability test at the temperature of 55°C., while its activity is still maintained.

The relationship between catalase activity and pH is illustrated in figure 2. The optimal of pH for the strain H37Rv is about 7.

In summary: The present findings indicate that the relations between the catalase activity of the strain *M. tuberculosis* H37Rv and temperature, as well as pH, are similar to those of other known catalases, provided a suitable method of investigation is applied.

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TUBERCULOUS POLYSEROSITIS

Polyserositis has been defined as a general inflammation of serous membranes with serous effusions. For the purpose of this paper the writers have included bilateral pleural effusion under polyserositis, although more common usage would perhaps exclude this category and include only those patients with combinations of pleural, pericardial, and peritoneal involvement.

The problem of differential diagnosis in some cases may be difficult, particularly in those patients with bilateral recurring effusion with definite or vague arthritic symptoms. The differential diagnosis might include tuberculosis, fungal infections, other infectious diseases, collagen disorders, blood dyscrasias, et cetera. In an attempt to gain insight into this problem an effort has been made to assemble cases of this syndrome. In doing this 22 cases attributable to tuberculosis were found and are the subject of this report.

In 1956, Acheson[1] presented an extensive review of the literature and noted that polyserositis was mentioned by several European physicians in the early nineteenth century. In 1846, Van Deen reported what was probably the earliest case history. Concato, in 1880, and Picchini, in 1891, described other patients in whom two or more serous membranes were involved. Pick discussed this syndrome primarily with regard to involvement of the pericardium with resulting circulatory embarrassment. Kelly, in 1903, referred to the clinical pattern as multiple serositis.

* * *

The case material for this report is based on 22 cases of polyserositis seen at Fitzsimons Army Hospital during a period of approximately four years.

In 11 patients, polyserositis was manifested by bilateral pleural effusions. Seven patients had pericardial fluid and involvement of one or both pleural spaces. Four patients had peritoneal involvement in association with effusion in one or both pleural spaces (table 1). Five of the 11 patients with bilateral pleurisy had no other demonstrable disease. Three had definite evidence of disseminated disease: one had miliary disease of the lung, one had meningitis, and one had renal and hepatic tuberculosis. Two had associated lymph node tuberculosis: one, of the cervical nodes; and one, of the inguinal lymph node. One patient had associated bilateral diffuse nodular disease of the apices of both lungs (table 2).

Symptomatology depended upon the site of greatest obvious involvement, and the duration of symptoms prior to seeking medical attention by the patient varied from several days to many weeks. Most patients complained of general malaise, fever, anorexia, and other symptoms of a generalized disease process at the time they were first seen by a physician and, in most instances, had pain in the chest, abdomen, or precordium, depending upon the site of the disease.

Information on aspirated fluid from effusions was available in 8 of the cases. In all, the specific gravity was 1.018 or higher, and the protein content was 3 gm. per 100 ml. or higher.

In 13 cases, acid-fast bacilli morphologically resembling *Mycobacterium tuberculosis* were seen or cultured from various specimens, including biopsies (table 3). In one case, acid-fast bacilli were seen in a biopsy specimen from the pleura; in another instance, the organisms were found in both a specimen from the pleura and a cervical lymph node. Cultures of sputum or gastric contents were positive for tubercle bacilli in 5 cases. Pleural fluid was the source of a positive culture for tubercle bacilli in one instance, while guinea pig inoculation with peritoneal fluid was positive in one. Tubercle bacilli were cultured from pericardial fluid in one case, from peritoneal biopsy material and a mesenteric lymph node in another, from sputum and urine in a third case, and from an inguinal node biopsy in another patient. Peritoneal biopsy was diagnostic in still another case.

The age of the patients ranged from twenty to fifty-eight, with only 2 patients more than thirty-one years of age. The fact that most of the patients were in the third decade of life and were males is in keeping with the fact that the majority of the patients at this hospital are drawn from young men in military service; however, reports of cases from civilian institutions indicate similar findings as to the age of highest incidence of tuberculous polyserositis.

Case analysis as to ethnic background revealed that 8 patients were U. S. Negroes, 4 were Asian in origin, one was Spanish-American, one was an American Indian, and 8 patients were classified as being of European descent. Twenty patients were males and 2 were females.

The tuberculosis chemotherapy employed in these cases consisted of various multiple-drug regimens of streptomycin, PAS, and isoniazid in standard doses administered from four to eighteen months and more. Even with this varied and, in some instances, inadequate therapy, all patients made excellent initial recoveries. One patient has had a known relapse. This man, who had bilateral pleurisy and peritonitis, received streptomycin and PAS for one hundred and twenty days along with eighteen months of hospitalization. He experienced a relapse four years later and developed far advanced bilateral pulmonary dis-

[1] ACHESON, R. M.: Quart. J. Med., 1956, *25*, 159.

TABLE 1

INVOLVEMENT OF SEROUS CAVITIES IN 22 CASES OF POLYSEROSITIS

Type of Effusion	
Bilateral pleural	11
Pericardial and pleural	7
Right pleural	3
Left pleural	2
Bilateral pleural	2
Peritoneal and pleural	4
Left pleural	2
Bilateral pleural	2

TABLE 2

EXTENT OF TUBERCULOSIS IN ELEVEN PATIENTS WITH BILATERAL PLEURAL EFFUSION

Pleural effusion only	5
and bilateral pulmonary tuberculosis	1
and miliary pulmonary tuberculosis	1
and tuberculous meningitis	1
and renal and hepatic tuberculosis	1
and cervical or inguinal node tuberculosis	2

TABLE 3

SITE OF ORIGIN OF BACTERIOLOGIC CONFIRMATION IN THIRTEEN CASES WITH TUBERCLE BACILLI DEMONSTRATED

Culture of sputum or gastric contents	5
Culture of urine	1
Culture of pleural fluid	1
Culture of peritoneal fluid	1
Biopsy: Peritoneal	2
Pleural	1
Cervical lymph node	1
Inguinal lymph node	1

ease. The follow-up observations of the other cases are too inadequate for comment.

* * *

Tuberculosis involves the serous membranes in a fashion similar to involvement elsewhere in the body, with the formation of tubercles. Not infrequently, an effusion may develop in one or more of the great serous sacs and polyserositis is noted. This fluid has been reported to have the characteristics of either a transudate or exudate in individual cases. In all of the present cases in which information was available, the fluid was an exudate. The fluid may be serous, serosanguineous, or sanguineous.

The work of Koch and von Pirquet helped demonstrate the concept of allergy to tuberculin. In 1917, Patterson noted that the pleural cavity of rabbits inoculated with virulent tubercle bacilli did not respond with the development of fluid. However, a second inoculation some weeks later produced an effusion. In 1936, Howard and DeVeer found that an experimental polyserositis could be produced by the introduction of tuberculin into one or more serous cavities of a previously sensitized guinea pig. From evidence in the present series, it is believed that, while allergy to tuberculoprotein might play some part, the latter is nearly always associated with definite tuberculous infection of the serous membranes.

The clinical features of tuberculous polyserositis include insidious onset, fever, chills, night sweats, anorexia, and pleural, pericardial, or abdominal pain, depending upon the serous cavities involved.

In the cases analyzed here, there was a rather high incidence of the disease in the patients of non-European descent. As in other reports, the age of highest incidence was in the second and third decades. In other series, a somewhat higher incidence of tuberculous polyserositis has been noted in males as compared with females. The difficulty of isolating tubercle bacilli from the fluid found in the serous sacs has been commented upon previously. In only 3 of the cases were tubercle bacilli cultured from fluid obtained from the pericardial, pleural, or peritoneal cavities.

Tuberculous polyserositis is generally believed to be not often associated with demonstrable hematogenous miliary spread, and in many proved cases previously reported there has been no evidence of disseminated miliary lesions at postmortem examination. However, Andrews and associates,[2] in their paper on this subject in 1948, indicated their belief that hematogenous spread was the most likely explanation of the appearance of polyserositis. Acheson, on the other hand, believes that this syndrome in tuberculosis is a manifestation of primary tuberculous infection and that the lymphatic system plays a major part in the pathogenesis. Spread to the pericardium may occur from a lymph node, to the peritoneal cavity from an infected organ or lymph nodes, and the pleura may react to a subpleural tuberculous focus. Many of the present patients showed evidence of disseminated disease, particularly those with peritoneal involvement or with bilateral pleural involvement. In those with pleural and pericardial involvement it is possible that some, at least, is caused by localized disease with lymph node erosion responsible for the pericardial disease.

In the literature the most dramatic complication reported in those patients who survived the acute illness was the development of con-

[2] ANDREWS, G. W. S., *et al.*: Quart. J. Med., 1948, *17*, 291.

strictive pericarditis. In the present series, made up of cases treated rather recently, no such complication has been noted as yet, although the follow-up observations are admittedly inadequate to this date. In the pre-chemotherapeutic era, miliary tuberculosis and tuberculous meningitis were also frequent causes of death.

The immediate prognosis in this type of tuberculosis is good with multiple-drug chemotherapy of adequate duration. It is the present practice to treat these patients with an isoniazid-containing regimen for a minimal period of eighteen months.

In summary: Twenty-two cases of tuberculous polyserositis are presented and the findings have been analyzed.

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INSTABILITY OF PENICILLIN IN DUBOS MEDIA

It is well known that penicillin is inactivated by a number of vehicles.[1-13] Benedict and associates[5] stated that it appeared that the destruction of penicillin in aqueous solution was a first-order irreversible reaction. Moreover, they found the optimal stability point for crystalline sodium penicillin in aqueous solution to be pH 6, and that the half-life of this drug at pH 6 was fourteen days at 24°C. The destructive effects of heavy metals such as copper and zinc on penicillin were reported by Smith.[8] Schwartzman[12] demonstrated the antagonistic effect of casein hydrolysate and asparagin on penicillin. Tompsett and associates[10] found that the activity of penicillin G was antagonized *in vitro* by the albumin fraction of serum. The rapid inactivation of penicillin by glycerin was demonstrated by Ferlauto and Clymer.[3]

In any test of antimicrobial activity, it is essential to determine the effect of the vehicle or the ingredients in the medium to be used on the activity of the antimicrobial to be tested. Dubos medium has been used in determining the *in vitro* effect of penicillin on the tubercle bacillus, but no attempt was made to determine quantitatively the effect of the medium on the activity of the penicillin.[14] With this principle in mind, the media of Dubos[14-16] have been studied, according to the method of Vincent and Vincent[17] with slight modifications, to determine quantitatively the effect of the various media on penicillin activity.

Test organism: Staphylococcus aureus (Upjohn strain) was used in all drug-susceptibility tests. It was transferred three times through 1 per cent tryptose broth before being used for bioassay.

Seeding plates: To 150 ml. of nutrient agar medium, 1.5 ml. of the twenty-four-hour tryptose broth culture of *Staphylococcus aureus* were added. Nine to ten heavy flat-bottomed Petri plates were then poured. The plates were allowed to set thirty minutes at room temperature to solidify before assay discs were placed on the surface.

Discs: Discs[18] (12.7 mm. in diameter) of highly absorbent, pure filter paper designed for the assay of penicillin were used. The discs were autoclaved and dried in the incubator for twenty-four hours before use.

Solutions assayed: Crystalline potassium penicillin G[19] dissolved in Dubos broth base medium containing Tween® 80 and Dubos medium albumin (20 ml. to 180 ml.).

Crystalline potassium penicillin G dissolved in Dubos broth base medium containing Tween 80 and Dubos oleic-albumin complex (20 ml. to 180 ml.).

These solutions were kept in the incubator at 37°C. until the time of bioassay.

Crystalline potassium penicillin G in phosphate buffer[20] at pH 6 and kept at 10°C. was used as a standard.

Method of assay: Using Vincent and Vincent's method,[17] test dilutions were made from a penicillin solution containing 100 units per ml. The discs were immersed in the test solutions for thirty seconds while being held with sterile forceps. Excess solution was then shaken off, and the disc was placed in position on the seeded plate. The final reading was made twenty-four hours later.

At twenty-four, forty-eight, and seventy-two hours, one week and two weeks, dilutions containing from 4 units per ml. to 0.05 units per ml. of penicillin were made anew from the original 100-unit per ml. tube of the Dubos medium.

The results were compared with those of a freshly prepared control consisting of a serial dilution from another vial of penicillin of the same lot number in a phosphate buffer[20] at pH 6 and kept at 10°C., which are optimal conditions for penicillin stability. The control dilutions contained from 0.10 units per ml. to 0.05 units per ml. of penicillin.

The per cent inactivation was determined by

[1] Fleming, A.: Brit. J. Exper. Path., 1929, *10* 226.

[2] Abraham, E. P., *et al.*: Lancet, 1941, *2*, 177.

[3] Ferlauto, R. J., and Clymer, H. A.: Science, 1947, *105*, 130.

[4] Randall, W. A., *et al.*: J. Am. Pharm. A. (Scient. Ed.), 1945, *34*, 110.

[5] Benedict, R. G., *et al.*: J. Bact., 1945, *49*, 85.

[6] Molinas, S., and Welch, H.: J. Am. Pharm. A. (Scient. Ed.), 1947, *36*, 41.

[7] Fisher, A. M.: Bull. Johns Hopkins Hosp., 1943, *73*, 343.

[8] Smith, E. S.: Quart. J. Pharm. & Pharmacol., 1946, *19*, 309.

[9] Clayton, J. C., *et al.*: Biochem. J., 1944, *38*, 452.

[10] Tompsett, R., *et al.*: J. Bact., 1947, *53*, 581.

[11] Bigger, J. W.: Lancet, 1944, *2*, 400.

[12] Schwartzman, G.: Science, 1945, *101*, 276.

[13] Schwartzman, G.: Science, 1945, *102*, 148.

[14] Kirby, W. M. M., and Dubos, R. J.: Proc. Soc. Exper. Biol. & Med., 1947, *66*, 120.

[15] Dubos, R. J., and Davis, B. D.: J. Exper. Med., 1946, *83*, 409.

[16] Dubos, R. J., and Middlebrook, G.: Am. Rev. Tuberc., 1947, *56*, 334.

[17] Vincent, J. G., and Vincent, H. W.: Proc. Soc. Exper. Biol. & Med., 1944, *55*, 162.

[18] Schleicher and Schuell & Co., Keene, New Hampshire.

[19] Abbott Laboratories, North Chicago, Illinois: Lot. No. 695-3611; expiration date December 1, 1960.

[20] Fisher, M. W.: Proc. Soc. Exper. Biol. & Med., 1954, *85*, 538.

TABLE 1

STABILITY OF CRYSTALLINE POTASSIUM PENICILLIN G IN DUBOS MEDIA AS COMPARED TO PHOSPHATE BUFFER pH 6 SOLUTIONS OF THE SAME LOT OF PENICILLIN

Vehicle	Storage Temperature	Per Cent Loss of Potency after Storage				
		24 Hours	48 Hours	72 Hours	7 Days	14 Days
Dubos broth base with Dubos medium albumin.	37°C.	0	0	50	90	95
Dubos broth base with Dubos oleic-albumin complex...	37°C.	50	99	99	95	96

comparing the diameters of the zones of inhibition of the test solutions with those of the standards.

The results of the bioassays are shown in table 1. Considerable loss of penicillin activity is to be noted. The Dubos medium containing the oleic-albumin complex showed the greatest inactivation. Inactivation in this medium could be observed within twenty-four hours. This difference is probably due to the presence of alkalinized oleic acid[5-8] in the latter medium.

* * *

It is clear from these results that Dubos broth base medium with the addition of Dubos oleic-albumin complex almost completely destroys penicillin (100 units per ml.) within forty-eight hours when inactivation was determined using *Staphylococcus aureus* as the test organism. Dubos broth base with the addition of Dubos medium albumin also destroys 50 per cent of the penicillin (100 units per ml.) after seventy-two hours, and 90 per cent after seven days of incubation.

The literature discloses that penicillin is unstable in the presence of several ingredients of the Dubos medium. They are: water;[4-6] glycerol,[2, 3] which is an ingredient of most media used for the culture of *M. tuberculosis*; certain minerals;[3, 9] albumin;[10, 11] asparagin and products of protein hydrolysis;[12, 13] and the environmental factors of pH, the optimal stability being at pH 6,[5-8] as well as temperatures above 10°C.[4-8]

These factors, which inactivate penicillin as incubation time increases, must be considered in the determination of the potency of antimicrobial drugs, especially against such organisms as *M. tuberculosis*.

The Oxford group[2] reported that penicillin did not inhibit the growth of *M. tuberculosis*. However, they used a broth containing glycerol. Kirby and Dubos[14] have cited contradictory reports in the literature regarding the effect of penicillin on the growth of *M. tuberculosis*. They found that a virulent human strain of *M. tuberculosis* is partially lysed by high concentrations of penicillin, and that small inocula (3 to 4 organisms per plate) are highly susceptible to concentrations of penicillin as low as 1 unit per ml. in the Dubos broth base with Dubos medium albumin. In the oleic-albumin complex medium, a concentration of 100 units per ml. caused no inhibition of growth.

The present observations show that, when penicillin is added to the Dubos oleic-albumin complex medium, the penicillin may not have inhibited the growth of *M. tuberculosis* because the medium destroyed the penicillin. Even the Dubos albumin medium destroyed the penicillin slowly during seven days of incubation.

Because of the slow growth of *M. tuberculosis* in conventional media, erroneous conclusions regarding the *in vitro* concentration of penicillin required to prevent the growth of the organism can be drawn. Before a valid conclusion regarding the true *in vitro* concentration of penicillin which will prevent the growth of this organism can be made, a medium must be found which will not destroy penicillin and which will promote the growth of the organism in the absence of penicillin. A review of the literature indicates that none of the conventional media would be suitable because they contain antipenicillin factors. Work is now in progress to find a method for assaying the susceptibility of *M. tuberculosis* to penicillin.

In summary: The Dubos broth base medium with the addition of oleic-albumin complex destroys penicillin relatively rapidly. Dubos broth base medium with the addition of the albumin complex slowly destroys penicillin during an incubation period of seven days. Dubos media are unsuitable for ascertaining the true susceptibility of *M. tuberculosis* to penicillin, unless accurate determinations are made to establish the amount of active penicillin remaining in the medium.

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HISTOPLASMA CAPSULATUM AND BLASTOMYCES DERMATITIDIS POLYSACCHARIDE SKIN TESTS ON HUMANS[1]

Problems concerning specificity and sensitivity of histoplasmin, blastomycin, and coccidioidin have been the stimuli to search for more efficient diagnostic skin test substances to be used for the detection of deep mycotic infections.

Hirsch and Benson[2] and Hirsch and D'Andrea[3] isolated by electrodialysis a polysaccharide-like substance from filtrates of *Coccidioides immitis*. With this substance they were able to produce skin test reactions in a patient infected with *C. immitis*. Using acetone, Van Pernis and coworkers[4] precipitated a polysaccharide substance from histoplasmin; this substance produced a positive skin test in a patient with histoplasmosis. Hassid and associates[5] isolated a polysaccharide from coccidioidin by precipitation with alcohol. This polysaccharide produced positive skin test reactions in infected persons. Peck and his coworkers[6] obtained a polysaccharide from both the mycelium and broth filtrates of cultures of *Blastomyces dermatitidis*. Using the polysaccharides, these investigators were able to produce typical skin reactions in the forearms of two patients with blastomycosis.

Previous studies from this laboratory[7] have shown that guinea pigs infected with *Histoplasma capsulatum* or *B. dermatitidis* gave positive skin test reactions when they were injected intradermally with the homologous polysaccharides. These polysaccharides offer the advantage of being employed in known amounts by weight. In this investigation, comparisons of *H. capsulatum* and *B. dermatitidis* polysaccharides were made with histoplasmin and blastomycin injected into human volunteers.

The Division of Biologic Standards of the National Institutes of Health has stipulated the requirements to be satisfied before marketing commercial histoplasmin. Unfortunately, similar regulations for the standardization of blastomycin and coccidioidin have not been published. The regulations necessary for the standardization of histoplasmin were followed in the preparation for human use of *H. capsulatum* strain G17(M) and *B. dermatitidis* strain 380 polysaccharides. The following tests were performed to comply with the regulations of the National Institutes of Health:

The *B. dermatitidis* and *H. capsulatum* polysaccharides, prepared from yeast-phase broth filtrates by methods previously described,[7] in a concentration of 1 mg. per ml., were each inoculated by streaking over the surface of two tubes of Sabouraud medium. This concentration of polysaccharide represents 100 times the amount employed in the skin tests. *B. dermatitidis* and *H. capsulatum* polysaccharides were each inoculated into duplicate tubes of thioglycollate broth. One thioglycollate and one Sabouraud culture from each of the two fungal skin test cultures were incubated at 37°C., while the other set of cultures was incubated at room temperature. These cultures were observed for twenty days before they were declared negative.

The same amount (1 mg. per ml.) of the *H. capsulatum* G17(M) and of *B. dermatitidis* 380 polysaccharides was inoculated intraperitoneally into a group of 20 mice. The mice were observed for toxic effects and deaths. After thirty days of observation the mice were sacrificed. Spleen and liver cultures, using blood agar containing antimicrobial drugs, were performed on all of the mice.

The *H. capsulatum* G17(M) and *B. dermatitidis* 380 polysaccharide solutions, in a concentration of 10 γ per 0.05 ml., were prepared and filtered through an ultra-fine sintered glass filter. Each of the skin test antigens was inoculated into two tubes of Sabouraud and two tubes of thioglycollate medium. One set of tubes was incubated at 37°C., and one set at room temperature. This procedure was necessary to determine the possible bacterial or fungal contamination of the solutions to be injected into humans.

Bacterial or fungal growth was not observed on any of the cultures. Mouse deaths were not observed, and the macroscopic and cultural examination of the mouse tissue failed to reveal the presence of *H. capsulatum* or *B. dermatitidis*. These tests were sufficient to comply with sterility regulations of the National Institutes of Health. The only other stipulation of these regulations was that the skin test preparation should be equal in skin reaction to the National Institutes of Health standard Lot No. 1 histoplasmin when tested in *H. capsulatum*-infected guinea pigs. The

[1] This investigation was supported, in part, by Research Grant E-922 from the Institute of Allergy and Infectious Diseases of the National Institutes of Health, U. S. Public Health Service; and, in part, by a research grant from the Office of Naval Research.

[2] Hirsch, E. F., and Benson, H.: J. Infect. Dis., 1927, *40*, 629.

[3] Hirsch, E. F., and D'Andrea, D.: J. Infect. Dis., 1927, *40*, 634.

[4] Van Pernis, P. A., *et al.*: J. A. M. A., 1941, *117*, 436.

[5] Hassid, W. Z., *et al.*: J. Biol. Chem., 1943, *194*, 305.

[6] Peck, R., *et al.*: J. Immunol., 1940, *38*, 449.

[7] Knight, R. A., and Marcus, S.: Am. Rev. Tuberc., 1958, 77, 983.

previous guinea pig skin tests had shown the *H. capsulatum* G17(M) polysaccharide to be equal to the National Institutes of Health Lot No. 1 histoplasmin, while the *B. dermatitidis* polysaccharide produced skin reactions equal to the available commercial blastomycin. Thus, according to the National Institutes of Health regulations, these products were safe for injection into humans.

The object of the human skin test experiment was to compare the reactivity of *H. capsulatum* G17(M) polysaccharide and *B. dermatitidis* 380 polysaccharide with histoplasmin and blastomycin.

The skin test reactions were performed on patient volunteers at the Veterans Administration Hospital in Salt Lake City. A group of persons from the University of Utah School of Medicine also volunteered for these injections. The left and right volar surfaces of the forearms of each person were injected as follows:

Left forearm:

(*a*) Upper portion: 0.1 ml. of 1:100 dilution of histoplasmin

(*b*) Lower portion: 0.05 ml. containing 10 γ of *H. capsulatum* G17(M) polysaccharide.

Right forearm:

(*a*) Upper portion: 0.1 ml. of 1:100 dilution of blastomycin

(*b*) Lower portion: 0.05 ml. containing 10 γ of *B. dermatitidis* polysaccharide.

Observations of the reactions were made at twenty-four and forty-eight hours. The extent of induration and erythema of each positive reaction was measured in millimeters and recorded. A history of geographic areas of residence was obtained from each person exhibiting a reaction of 5 mm. or more of induration.

TABLE 1

HUMAN SKIN TEST REACTIONS

COMPARISON OF H. CAPSULATUM STRAIN G17(M) POLYSACCHARIDE WITH COMMERICAL HISTOPLASMIN.

Histoplasmin positive, polysaccharide positive	16
Histoplasmin positive, polysaccharide negative	2
Histoplasmin negative, polysaccharide positive	2
Histoplasmin negative, polysaccharide negative	114
Total positive reactions	20
Total number of tests	134
Per cent positive	15.7

All persons with positive skin tests have histories of having lived in an *Histoplasma capsulatum* endemic area.

TABLE 2

HUMAN SKIN TEST REACTIONS

COMPARISON OF B. DERMATITIDIS STRAIN 380 POLYSACCHARIDE WITH COMMERCIAL BLASTOMYCIN

Blastomycin positive, polysaccharide positive	0
Blastomycin positive, polysaccharide negative	0
Blastomycin negative, polysaccharide positive	5
Blastomycin negative, polysaccharide negative	129
H. capsulatum polysaccharide positive, *B. dermatitidis* polysaccharide positive	5
Histoplasmin positive, *B. dermatitidis* polysaccharide positive	0

TABLE 3

HUMAN SKIN TEST REACTIONS AMONG 20 POSITIVE REACTORS

	Mean Diameter of Induration in Mm.	
	24 Hours	48 Hours
Histoplasmin	6.9	12.4
H. capsulatum polysaccharide	12.8	14.0
Blastomycin	0.33	0
B. dermatitidis polysaccharide	9.2	7.0

* * *

Skin tests have been performed on 134 persons. A comparison of the reactivity of the histoplasmin and the *H. capsulatum* G17(M) polysaccharide at forty-eight hours is shown in table 1. In all, 20 positive reactions were noted.

A summary of the comparative reactivity of the blastomycin and the *B. dermatitidis* 380 polysaccharide can be seen in table 2. The only reactions noted were nonspecific, that is, less than 5 mm. in extent. The mean diameter of induration for each of the skin testing antigens is summarized in table 3.

* * *

Previous guinea pig studies[7] with infected animals revealed that the *H. capsulatum* G17(M) polysaccharides produced a mean skin test reaction of 13 mm. Among humans who reacted in this study, a mean skin test reaction of 14 mm. was observed. The comparison of the human skin test reactions to the histoplasmin and the *H. capsulatum* G17(M) polysaccharide in 10 γ amounts

appears to be about equal. It is interesting that the skin test reaction of the polysaccharide was almost maximal at twenty-four hours, while the histoplasmin showed maximal reactivity at forty-eight hours.

All of the persons exhibiting positive histoplasmin skin tests had spent periods of time in the known histoplasmosis endemic area of the Mississippi River valley.

Previous guinea pig skin tests with *B. dermatitidis* 380 polysaccharide showed no cross-reactions in guinea pigs infected with *H. capsulatum*, while the commercial blastomycin produced a significant number of cross-reactions. In humans, the situation seems to have reversed itself, since blastomycin showed no cross-reactions, while a mean 7 mm. of induration of nonspecific reactions was noted with *B. dermatitidis* 380 polysaccharides. However, the commercial blastomycin was inferior to the *B. dermatitidis* polysaccharide in homologous skin tests in *B. dermatitidis*-infected guinea pigs. Human blastomycosis infections at the present time are very infrequent in this part of the country; therefore the opportunity for a comparative study of blastomycin and *B. dermatitidis* polysaccharides is not feasible.

In summary: Skin tests were carried out on 134 persons injected with histoplasmin, blastomycin, and polysaccharides derived from broth in which the yeast phase of *H. capsulatum* and *B. dermatitidis* was grown. The histoplasmin and the *H. capsulatum* polysaccharide were equally sensitive. The *B. dermatitidis* polysaccharide yielded 5 positive reactions among persons exhibiting a concomitant histoplasmin sensitivity. The relative specificity of these reactions could not be determined.

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February 24, 1959

A NEW LIQUID SYNTHETIC MEDIUM FOR THE CULTIVATION OF MYCOBACTERIUM SPECIES

M. tuberculosis has been grown on synthetic medium since the studies of Proskauer and Beck[1] in 1894. Certain minerals (P, S, K, and Mg.) have been found to be essential in the medium.[2] The addition of other minerals, although not essential for growth, may give favorable results. Long and Seibert[3] obtained an increase of 7.3 per cent growth by the addition of ferric ammonium citrate in Long synthetic medium. Fisher[4] suggested that hemin might be a growth factor for certain isoniazid-resistant strains. Many carbohydrates have been investigated as carbon sources, but glycerol and glucose have been the most widely used.

A medium which will support the growth of various strains of *Mycobacterium tuberculosis*, and at the same time is chemically defined, has been formulated in this laboratory. It contains the essential minerals (P, S, K, and Mg.) plus a very small amount of ferric ammonium citrate. It contains ammonium chloride as a source of nitrogen and glucose as a carbon source. It also contains Tween® 80 as a surface tension depressant. The basal medium is prepared according to the formula shown in table 1. It is adjusted to pH 6.8 and sterilized in an autoclave for fifteen minutes at 15 pounds' pressure (121°C). Following sterilization, 20 ml. of sterile 50 per cent glucose solution are added to the basal medium aseptically.

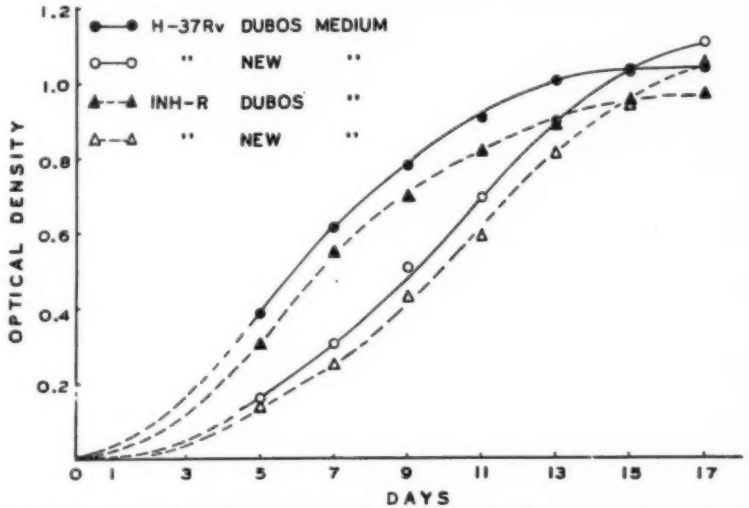

FIG. 1. Typical turbidimetric growth curves comparing the growth of two strains of *Mycobacterium tuberculosis* in Dubos Tween-albumin medium and the new synthetic medium described. The isoniazid-susceptible strain was H37Rv supplied by Dr. William Steenken, Jr., of the Trudeau Laboratory, and the isoniazid-resistant strain was locally isolated.

This medium was devised for the purpose of studying growth by turbidimetric measurements. It was shown by Monod[5] that the opacimetric measurement corresponds to the weight of the microbial substance. The medium has two advantages for spectrophotometric measurements: (*1*) the basic medium is clear; the light transmission approaches that of water, and (*2*) the addition of Tween-80 as a surface tension depressant provides finely dispersed subsurface growth. This medium has an advantage over other liquid media

[1] PROSKAUER, B., AND BECK, M.: Ztschr. f. Hyg., 1895, *18*, 128.

[2] SAUTON, B.,: Compt. rend. Acad. Sc., 1912, *155*, 860.

[3] LONG, E. R., AND SEIBERT, F. B.: Am. Rev. Tuberc., 1926, *13*, 393.

[4] FISHER, M. W.: Am. Rev. Tuberc., 1954, *69*, 797.

[5] MONOD, J.: Ann. Rev. Microbiol., 1949, *3*, 371.

TABLE 1

FORMULA OF SYNTHETIC MEDIUM FOR MYCOBACTERIA

Potassium phosphate, monobasic	5.0	gm.
Ammonium chloride	5.0	gm.
Magnesium citrate	1.0	gm.
Potassium sulfate, dibasic	0.5	gm.
Tween 80, certified	0.2	ml.
Ferric ammonium citrate	0.0025	gm.
Distilled water to make	980.0	ml.
Adjust to pH 6.8 with potassium hydroxide, sterilize, cool, add sterile 50 per cent glucose	20.0	ml.

such as the Tween-albumin medium of Dubos and Middlebrook[6] for spectrophotometric readings because of its lack of color. The Dubos medium has a pale yellow color, which may vary during the period required for a growth curve. Two or three glass beads are placed in each tube of medium to aid in evenly dispersing the growth before spectrophotometric readings are taken. Because of the subsurface growth, aeration of the cultures will increase the yield and lower the generation time. Aeration is obtained by manual agitation of the cultures at regular intervals during growth. A mechanical shaker can be used, such as that demonstrated by Miller and Roessler.[7] They showed that agitation during growth produced more rapid division and greater cell numbers. This medium can be applied as a tool in a number of research problems. It is not suitable for use as a diagnostic growth medium. Since it is chemically defined and quite simple, it is applicable to many growth and nutrition studies. The medium contains only one source of nitrogen, which is inorganic, and one source of carbon, thereby providing good conditions for accurate determinations and more precise interpretations of the findings in studies of metabolic processes.

Typical growth curves obtained with this medium and with Dubos medium[6] are shown in figure 1.

In summary: A new liquid synthetic medium has been devised which is chemically defined with one inorganic nitrogen source and one carbon source; is clear and colorless; permits subsurface growth; and is readily adaptable to nutritional and metabolic studies.

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[6] DUBOS, R. J., AND MIDDLEBROOK, G.: Am. Rev. Tuberc., 1947, *56*, 334.

[7] MILLER, I. L., AND ROESSLER, W. G.: Am. Rev. Tuberc., 1956, *73*, 716.

EVALUATION OF CYCLOSERINE WITH HIGH DOSAGE OF ISONIAZID IN CHRONIC TREATMENT-FAILURE PULMONARY TUBERCULOSIS[1]

Management of the chronic, active, treatment-failure case continues to be a major problem in tuberculosis hospitals. Disappointing results have attended the trials of many secondary drug regimens. Most of the cycloserine studies have reported a low rate of sputum "conversion," a high rate of relapse, and confusing data concerning drug resistance.

The present study was designed to determine the effectiveness of cycloserine administered with a high dosage of isoniazid when compared with cycloserine alone and with cycloserine along with an ordinary dosage of isoniazid. A major effort was made to evaluate the importance of *in vitro* bacillary resistance to cycloserine.

Forty-five patients with chronic, far advanced, sputum-positive pulmonary tuberculosis were selected. All of them had received streptomycin, isoniazid, and para-aminosalicylic acid (PAS) in various combinations for several years. In addition, 7 had been given pyrazinamide and 5, viomycin. Many had had collapse procedures and 5 had undergone pulmonary resection prior to the study. None had received cycloserine nor were any considered to be candidates for surgery because of the extent of disease, limited pulmonary function, or both. The average age was fifty-two years.

Three regimens of oral therapy were used: cycloserine[2] alone, 250 mg. twice daily; the same dosage of cycloserine plus isoniazid, 150 mg. twice daily; the same dosage of cycloserine plus isoniazid, 500 mg. twice daily, plus 100 mg. of pyridoxine twice daily.

The patients were assigned to therapy as their names appeared on an alphabetic list except that the groups were equalized as far as possible with respect to cases of progressive disease and patients with strains of tubercle bacilli with lesser degrees of resistance to isoniazid.

Thirty-five patients either completed nine to thirteen months of treatment or died sometime after the second month. Eleven had progressive disease, while in 24 the disease had been stationary for at least one year. Twenty-two patients excreted tubercle bacilli which were isoniazid resistant and catalase negative.

Sputum smears and cultures were done at monthly intervals, and susceptibility to cycloserine and isoniazid was determined on all positive cultures. Subcultures in liquid Tween®-albumin medium were tested by spreading a loopful of the culture on slants or quadrant plates of Middlebrook oleic-acid–albumin agar containing the following concentrations of drugs in γ per ml.: cycloserine—25, 40, 80; isoniazid—0.2, 1.0, 10. As cycloserine becomes inactivated readily, the medium was always freshly prepared, and a control culture of H37Rv was included to check the inhibitory activity of the drug medium. Tests were observed for fourteen to twenty days.

Drug toxicity: Toxic effects were noted only in the patients receiving cycloserine and the high dosage of isoniazid. In 3 of the original 14 patients in this group, it was necessary to discontinue treatment or to change to another regimen. One patient was dropped from the study after one month because of an episode of convulsions and stupor thought to be associated with excessive alcoholic intake. Another patient developed acute depression and paranoid reaction after three weeks of therapy. He recovered and remained well mentally after he was changed to the lower isoniazid dosage. After fourteen weeks of treatment, the third patient suddenly became extremely agitated and belligerent. His mental state improved promptly with the aid of tranquilizers, and therapy was continued with cycloserine alone. The synergistic toxicity of cycloserine with high dosage of isoniazid was noted earlier by Biehl.[3] Periodic blood counts, urinalyses, and liver function tests showed no abnormalities throughout the study.

Therapeutic results: The results of therapy are summarized in table 1. Eight of the 35 patients derived at least temporary benefit; in 5 patients the progression of disease was halted, and in 3 others improvement was noted. Of the latter, one patient (cycloserine–high dosage isoniazid group) who had progressive disease prior to the study gradually became afebrile, coughed less, and achieved and maintained roentgenographic stability for thirteen months although her sputum continued to be positive for tubercle bacilli. The other 2 (cycloserine-isoniazid group), each with stationary disease beforehand, showed no appreciable roentgenographic change but did have definite improvement clinically and bacteriologically. Only one of them, however, "converted" her sputum cultures to "no growth" on at least two successive occasions. Unfortunately, both of these

[1] This study was aided by a grant from the Anti-Tuberculosis League of Cleveland and Cuyahoga County.

[2] Cycloserine was supplied as Seromycin® by Eli Lilly and Co.

[3] BIEHL, J. P.: Tr. Sixteenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration-Armed Forces, 1957, p. 58.

TABLE 1
OVER-ALL RESULTS OF TREATMENT

Regimen	Status of Disease Prior to Study	Number of Patients	Improved	No Change	Worse	Died
Cycloserine alone	Progressive	4	0	1	0	3 (1)*
	Stationary	7	0	4	1	2 (2)
	Total	11	0	5	1	5 (3)
Cycloserine-isoniazid	Progressive	2	0	1	0	1 (1)
	Stationary	10	2†	7	1	0
	Total	12	2	8	1	1 (1)
Cycloserine–high dosage isoniazid	Progressive	5	1‡	3	1	0
	Stationary	7	0	7	0	0
	Total	12	1	10	1	0
All regimens	Progressive	11	1‡	5	1	4 (2)
	Stationary	24	2†	18	2	2 (2)
	Total	35	3	23	3	6 (4)

* Figures in parentheses indicate number of deaths from massive hemoptysis.
† Both subsequently relapsed after nine and ten months of treatment, respectively.
‡ Improvement confined to diminished cough and gradual loss of fever.

patients subsequently had clear-cut relapses of disease while still on therapy, at nine months and ten months, respectively. There were no closures of cavity or any other instances of sputum "conversion."

Three patients (one in each treatment group) showed progression of disease roentgenographically without prior improvement, and 6 patients died after the second month of treatment. Four of the deaths were due to sudden massive hemoptysis; the other 2 followed steadily progressive disease uninfluenced by cycloserine. Two of the roentgenographic increases occurred in patients with previously stationary disease, one at the fourth month and the other at the eighth month of therapy.

The small number of patients in each group prevents valid inter-regimen comparisons. No appreciable difference was noted between the two multiple-drug regimens. The results of therapy with cycloserine alone, however, seemed to be inferior since more than half of the patients experienced disease progression or died, in contrast to the 13 per cent in the two multiple-drug regimens whose disease showed deterioration or who died. When the deaths due to hemoptysis are discounted, the difference was 27 per cent versus 8 per cent. In addition, there was only one instance of improvement or halt of progression of disease among the 11 patients treated with cycloserine alone, while there were 7 such instances (29 per cent) among the 24 patients in the multiple-drug groups.

Drug resistance: The results of cycloserine susceptibility tests are summarized in tables 2 and 3. Roughly 20 per cent of the pretreatment cultures were only partially inhibited by 25 γ, and one culture was able to grow on the 25-γ quadrant almost as well as on medium without drug. The percentage of cultures slightly resistant to 25 γ did not increase during treatment.

Evidence of increased resistance was noted after the second month of treatment. Considering as resistant those cultures showing good growth in 25 γ, there were less than 5 per cent resistant prior to treatment, 27 per cent resistant after two to four months, and from 31 to 40 per cent resistant thereafter. Only four cultures (two each at the six- to eight- and eight- to thirteen-month periods, respectively) were able to grow on the 40-γ quadrant. In each case the growth was distinctly less than that on the control quadrant. No difference was seen in the resistance pattern among the three treatment groups.

In cultures from the same patient, it was not uncommon to note fluctuation from month to month between no growth and moderate growth on the 25-γ quadrant. In table 3 the patients are classified into those whose cultures remained completely susceptible, those who showed intermittent resistance to 25 γ, and those who showed consistent ability to grow on the 25-γ quadrant. Thirty-eight per cent of the patients for whom adequate data were available consistently excreted bacilli resistant to 25 γ. A greater tendency to excrete resistant bacilli was noted in the patients who had progressive disease prior to the study. Patients who excreted bacilli which grew slightly in the lowest drug concentration prior to therapy did not demonstrate subsequently a higher incidence of drug resistance than those patients whose cultures were completely susceptible.

Persistence of cycloserine resistance after therapy:

TABLE 2

EMERGENCE OF CYCLOSERINE RESISTANCE IN SERIAL CULTURES FROM TREATED PATIENTS

Drug Regimen	Patient-Strains Tested	Pretreatment	1 to 2 Months	2 to 4 Months	4 to 6 Months	6 to 8 Months	8 to 13 Months
Cycloserine alone	Total	10	9	9	8	5	4
	Susceptible to 25γ	8	8	4	4	2	2
	Partially resistant to 25γ	1	0	2	1	1	1
	Resistant to 25γ	1	1	3	3	2	1
	Partially resistant to 40γ	0	0	0	0	0	0
Cycloserine-isoniazid	Total	9	11	10	12	10	6
	Susceptible to 25γ	8	8	5	6	5	2
	Partially resistant to 25γ	1	3	2	1	1	3
	Resistant to 25γ	0	0	3	5	2	0
	Partially resistant to 40γ	0	0	0	0	2	1
Cycloserine–high dosage isoniazid	Total	8	9	10	10	8	8
	Susceptible to 25γ	6	6	6	5	4	2
	Partially resistant to 25γ	2	3	2	1	3	1
	Resistant to 25γ	0	0	2	4	1	4
	Partially resistant to 40γ	0	0	0	0	0	1
All regimens	Total	27	29	29	30	23	18
	Susceptible to 25γ	22 (82%)	22 (76%)	15 (52%)	15 (50%)	11 (48%)	6 (33%)
	Partially resistant to 25γ	4 (15%)	6 (21%)	6 (21%)	3 (10%)	5 (22%)	5 (28%)
	Resistant to 25γ	1 (4%)	1 (3%)	8 (27%)	12 (40%)	5 (22%)	5 (28%)
	Partially resistant to 40γ	0	0	0	0	2 (9%)	2 (11%)

TABLE 3

PATIENTS WHOSE CULTURES SHOWED CYCLOSERINE RESISTANCE AT ANY TIME

	Number of Patients*	Remained Susceptible to 25γ per/Ml.	Showed Intermittent Resistance to 25γ per/Ml.	Showed Consistent Resistance to 25 or More γ per/Ml.
Cycloserine alone	8	4	1	3 (38%)
Cycloserine-isoniazid	11	4	2	5 (45%)
Cycloserine–high dosage isoniazid	10	5	2	3 (30%)
All regimens	29	13	5	11 (38%)
Total, progressive disease pretreatment	9	3	1	5 (56%)
Total, stable disease pretreatment	20	10	4	6 (30%)

* Includes only those for whom satisfactory data are available for at least six months of therapy.

In table 4 is shown the cycloserine susceptibility of post-therapy cultures from 11 patients whose bacilli had demonstrated drug resistance during treatment. It will be noted that the follow-up cultures tended to retain drug resistance for a few months, but that most of the patients were excreting drug-susceptible bacilli after one year.

In table 4 one can also see the variation in susceptibility which characterized the serial tests from some patients.

Relationship between clinical results and drug resistance: The clinical results according to drug resistance status are outlined in table 5. Only those patients who were adequately studied for at least six months are included. There was a suggestive association between drug resistance and poor outcome, although in the 2 instances of definite relapse after initial improvement neither could be related to the emergence of *in vitro* resistance to cycloserine. Interestingly enough, the cultures from one of these patients (cycloserine-isoniazid group) showed an increase in isoniazid

TABLE 4

STABILITY OF CYCLOSERINE RESISTANCE OF TUBERCLE BACILLI ISOLATED DURING AND AFTER TREATMENT

Patient	Pretreatment	During Treatment			Post-treatment		
		2 to 4 Months	4 to 8 Months	8 to 13 Months	1 to 4 Months	4 to 6 Months	12 to 16 Months
PK	S	R25	R25	R25	PR40	—	—
GB	S	R25	R25	—	R25	S	—
MK	R25	S; R25	PR25	PR25	S	—	S
AD	S	S	R25; S	PR40	PR40	—	PR25
HO	—	PR25	R25	—	R25	R25	S
AJ	S	R25	R25; PR40	—	PR40	PR25	S
FH	S	S	S; R25	—	PR25	R25	S
JM	S	R25	S; R25	PR25	—	PR25	S
JM	S	R25	R25	R25	PR40	—	—
MH	—	—	R25	PR40	PR40	—	R25
JB	S	S	S	R25	R25	—	—

Abbreviations: S = susceptible to 25 γ per ml.; R = resistant; PR = partially resistant. Figures indicate γ of cycloserine per ml. of medium.

TABLE 5

RELATIONSHIP OF IN VITRO DRUG RESISTANCE AND CLINICAL RESULT

Drug Resistance Status	Number of Patients	Improved	Improved Then Relapsed	No Change	Worse or Died
Remained susceptible to 25γ per ml.	13	0	1	11	1
Showed intermittent resistance to 25γ per ml.	5	1	1	3	0
Showed consistent resistance to 25γ per ml. or more	11	0	0	6	5

resistance at the time of relapse. On the other hand, both of the patients with previously stationary disease who became worse during treatment began consistently to excrete bacilli resistant to 25 γ of cycloserine within a month of this event.

Changes in isoniazid resistance: Prior to the study, 11 patients excreted tubercle bacilli which were still susceptible to 10 γ of isoniazid per ml. of medium despite prolonged therapy with this drug. Two of these "less resistant" strains were inhibited by as little as 1 γ per ml. Cultures from the 3 patients treated with cycloserine alone all maintained their original isoniazid resistance. Cultures from one patient in each of the two multiple-drug regimen groups demonstrated an increase in isoniazid resistance. Originally resistant to 1 γ, susceptible to 10 γ, and catalase deficient, both of these strains became resistant to 10 γ of isoniazid at the eighth to ninth month of treatment. One of these patients showed a coincident relapse of disease, as stated previously.

Worthy of special mention is the case of *WW*, whose cultures were catalase positive and consistently inhibited by 1 γ of isoniazid despite previous long-term administration of this drug. Bioassay of his serum after ingestion of 500 mg. of isoniazid and 4 gm. of PAS revealed concentrations of 4.8 and 1.2 γ of isoniazid per ml. at two and six hours, respectively. With these adequate serum concentrations, however, his cultures retained the original level of isoniazid resistance during one year on the cycloserine–high-dosage-isoniazid regimen.

* * *

The results of this study indicate that the higher dosage of isoniazid was no more beneficial than ordinary dosage of the drug when each was given in combination with cycloserine. The increased toxicity of the cycloserine–high-dosage-isoniazid regimen should, therefore, limit its use to selected cases in which there is rapid inactivation of isoniazid.

A relatively low dosage of cycloserine was administered to the patients in this study. Recent reports[4-6] suggest that better results may be ob-

[4] PYLE, M. M., *et al.*: Presented at the Eighteenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration-Armed Forces, 1959.

tained by larger doses, although special measures must be undertaken to combat the increased toxicity. It should be recalled that the cycloserine concentrations of 15 to 30 γ per ml. attained in the blood with an oral dose of 250 mg. are not appreciably higher than the 10 to 20 γ per ml. necessary to inhibit growth of mycobacteria in the test tube. It is logical to assume that better results would follow if the quantity could be made to reach higher concentrations. The narrow margin between the concentrations maintained in the patient's blood and the minimal inhibitory concentration may also explain how a small increase in bacillary resistance could be associated with clinical relapse.

The increase of bacillary resistance to cycloserine during treatment is characteristically of small magnitude, usually not exceeding eightfold, in contrast to the hundred-fold increments commonly observed to streptomycin, isoniazid, and PAS. Resistance to cycloserine also emerges very slowly *in vitro*[7] but, lest it be assumed that such a combination of *in vitro* and *in vivo* observations is the rule, it should be pointed out that emergence of resistance to PAS is also very gradual in the test tube but may be abrupt and of considerable magnitude in the treated patient.

The instability of cycloserine makes the interpretation of minor degrees of change in susceptibility difficult to evaluate unless the tests are scrupulously controlled. In the present study it was learned that oleic-acid–agar medium with cycloserine loses appreciable activity after just a few days in the refrigerator. It would be more meaningful to report cycloserine susceptibilities in terms of a "resistance ratio" using a standard strain (H37Rv) as reference in each series of tests, as is the custom in some English laboratories.[8]

In summary: Thirty-five patients with chronic, active, "treatment-failure" pulmonary tuberculosis were divided into three groups and treated for nine to thirteen months with either cycloserine alone, cycloserine plus ordinary dosage of isoniazid, or cycloserine plus high dosage of isoniazid. The dosage of cycloserine was 250 mg. twice daily. General improvement was noted in 3 patients, and the progression of disease was halted, at least temporarily, in 5 others. Two of the former, however, relapsed while still on treatment and, in addition, 9 patients became worse or died while on therapy. Elimination of cultivable tubercle bacilli from the sputum occurred in only one patient, but even in this case the sputum became positive again a few months later.

Better results were observed with the multiple-drug regimens than with cycloserine alone, but the larger isoniazid dosage seemed to offer no advantage over the lower isoniazid dosage. Significant toxicity was observed only in the cycloserine–high-dosage-isoniazid group.

Serial cultures from 38 per cent of the patients showed an increase of resistance to cycloserine. The increase was restricted to a two- or fourfold difference. In most instances it could be demonstrated that this degree of bacillary resistance persisted for several months after treatment was stopped, but one year post therapy only 2 of the 7 patients tested continued to excrete drug-resistant bacilli. It was not possible to demonstrate a clear-cut relationship between *in vitro* resistance and progression of disease.

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[5] RITCHIE, J. A., *et al.*: Tubercle, 1958, *39*, 289.
[6] EPSTEIN, I. G., *et al.*: Antibiotics Annual, 1958–59, Medical Encyclopedia, Inc., New York, 1959, p. 472.
[7] STEENKEN, W., JR., AND WOLINSKY, E.: Am. Rev. Tubere., 1956, *73*, 539.

FURTHER OBSERVATIONS ON THE BIOLOGIC ACTIVITY OF CRUDE MYCOBACTERIAL CELLS[1]

Developments during recent years in the chemotherapy of tuberculosis have led to general recognition of the fact that mechanisms for increasing host resistance must be developed before true eradication of the infection will be achieved. The simultaneous gradual decrease in the prevalence of tuberculin reactors among the general population in certain areas has led to realization of the potential practical value of the tuberculin reaction in the detection of infected persons. Thus, studies designed to develop immunizing agents capable of conferring protective immunity without simultaneously producing skin hypersensitivity to tuberculoprotein were initiated by Weiss and Dubos in 1955.[2]

A technique for the mass cultivation of the Phipps BCG strain of tubercle bacilli was subsequently developed by the present writers and was described in a recent publication.[3] Evidence was presented, moreover, which indicates that such cells, when grown *en masse* and subsequently phenolized and lyophilized, are as capable of protecting animals against challenge with virulent strains of *Mycobacterium tuberculosis* as are phenolized nonliving BCG cells grown under more conventional conditions.[3, 4]

In the production of any vaccine or immunizing agent, large numbers of microbial cells necessarily must be cultivated. Thus, it is technically advantageous to employ an attenuated strain. The possibility that greater protection might be obtained with virulent strains, however, must be considered.

In the present report, data will be presented which show the relative protective power of: the Phipps BCG strain of bovine tubercle bacilli, an attenuated strain; the Vallée strain of bovine tubercle bacilli, a virulent strain; and the H37Rv strain of human tubercle bacilli, also a virulent strain.

Unextracted, nonliving, lyophilized BCG cells were used throughout the study. In all instances, the crude cells were injected subcutaneously into mice three weeks prior to intravenous challenge with the Vallée bovine strain of *M. tuberculosis*. In some instances, multiple, i.e., 2 or 3, immunizing doses of cells were administered; in such instances, the immunizing doses were administered seven days apart, and animals were challenged three weeks after the last immunizing dose.

In figure 1, the average survival time of animals immunized with BCG, H37Rv, or Vallée cells and subsequently challenged, as described above, is shown during a thirty-one-day observation period. From the data presented, it is apparent that, with the particular lots of BCG cells used in this set of experiments, increasing the size of the immunizing dose from 0.3 to 0.9 mg. increased the protective effect. Even with a 0.9-mg. dose, however, only a slight increase in average survival time over that observed with the nonimmunized controls was observed. Moreover, although in all three sets of data the Vallée cells increased the average survival time to the greatest extent, no significant difference was observed between the 3 strains.

As shown in figure 2, multiple small doses of H37Rv or of Vallée cells were more effective, however, than single doses of these cells. In addition, these 2 strains were more effective than the BCG strain in this particular series of experiments.

The effectiveness of multiple small doses of the Vallée and H37Rv cells was not significantly greater than that of a single large dose of BCG cells (figure 3.) Large amounts of BCG cells can not always be administered, however, due to the presence of "sensitizing" substances in some batches of cells. The presence of such a substance, or of such substances, in crude BCG cells and their ability to interfere with demonstration of their protective power have been discussed elsewhere [4, 5] The results obtained in the present series of experiments suggest that such a substance or substances may also be present at times in batches of H37Rv and Vallée cells.

The quantity of that substance responsible for the protective effect likewise varies from one lot of cells to another. Until at least partially purified fractions of cells become available, therefore, no firm conclusions can be drawn as to the merits of employing virulent vs. attenuated strains for development of a cell-free immunizing agent.

[1] Presented in part at the Eighteenth Conference on the Chemotherapy of Tuberculosis, held under the auspices of the U. S. Veterans Administration-Armed Forces, St. Louis, Missouri, February, 1959.

[2] Weiss, D. W., and Dubos, R. J.: J. Exper. Med., 1955, *101*, 313.

[3] Lenert, T. F., *et al*: Am. Rev. Tuberc., 1958, *78*, 934.

[4] Hobby, G. L., *et al.*: Am. Rev. Tuberc., 1958, *78*, 939.

[5] Dubos, R. J., and Schaedler, R. W.: J. Exper. Med., 1957, *106*, 703.

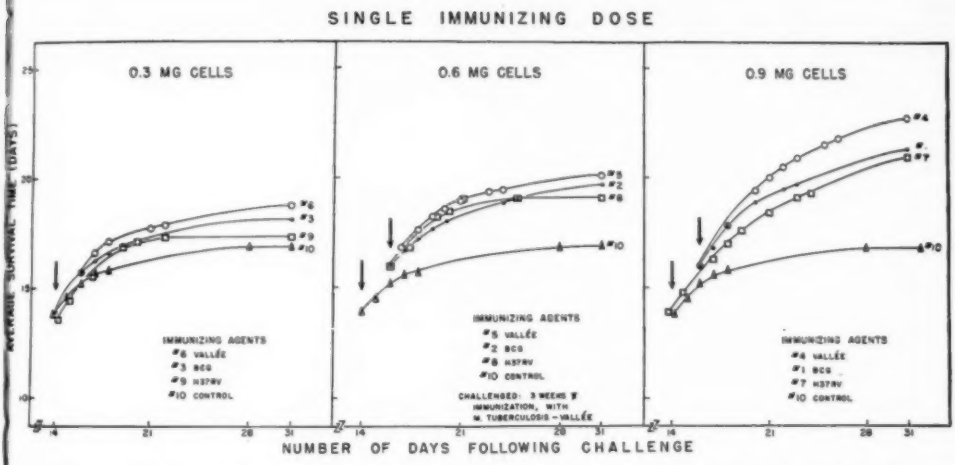

FIG. 1. Average survival time of animals immunized with BCG, H37Rv, and Vallée cells during thirty-one day observation period.

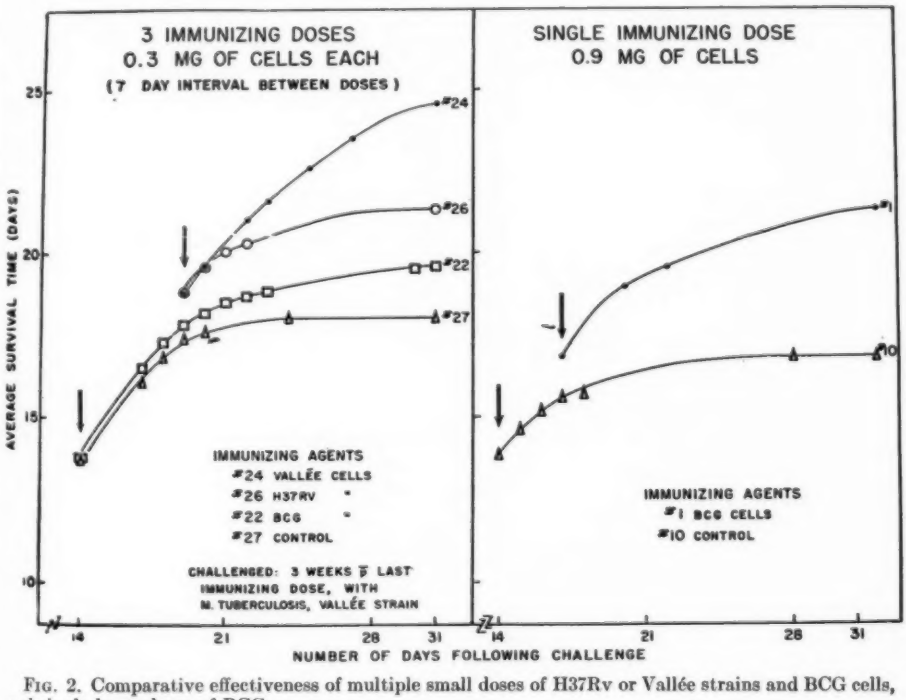

FIG. 2. Comparative effectiveness of multiple small doses of H37Rv or Vallée strains and BCG cells, and single large doses of BCG.

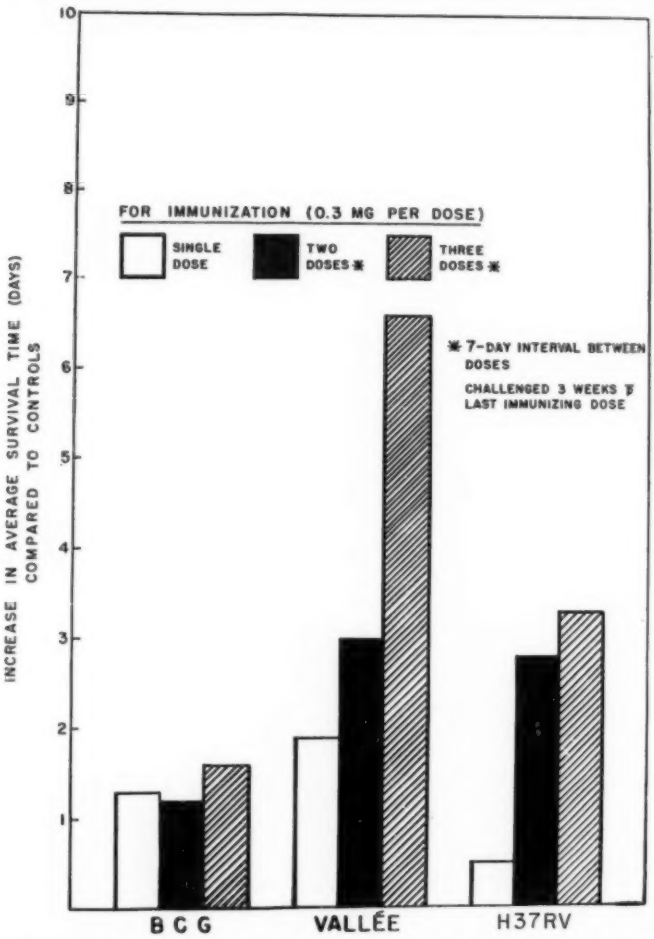

FIG. 3. Increase in survival time calculated at thirty-one days after challenge with Vallée strain of *M. tuberculosis*. Comparison of single and multiple doses of the immunizing dose of BCG, Vallée, or H37Rv cells.

In summary: Unextracted, nonliving, lyophilized BCG cells were more effective as immunizing agents against tuberculous infections of mice when administered in a dosage equivalent to 0.9 mg. of cells than when administered in smaller quantities. The degree of protection resulting from the administration of single large doses of BCG cells, moreover, was as great as that produced by multiple small doses of nonliving lyophilized H37Rv or Vallée cells. These data suggest that dosage and mode of administration of the immunizing doses may be of greater importance than the strain employed for such immunization. In addition, they suggest that, when appropriate conditions are used, killed mycobacterial cells can produce as good protection as has been reported with live BCG.

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SOURCE OF SCOTOCHROMOGENS

To the Editor of The American Review of Tuberculosis and Pulmonary Diseases:

Some chromogenic acid-fast bacilli occurring in gastric lavage specimens or sputum are probably of no significance. That these mycobacteria lack importance in human disease has been recognized for decades; accordingly, in the past their presence has rarely been reported. Currently there is a tendency to ascribe significance to them. One hypothesis suggests a causal relationship between the antimicrobial treatment of tuberculosis and the emergence of these scotochromogens.

Scotochromogenic acid-fast bacilli are of more than one kind. The commonest one found in clinical laboratories is of yellow-orange color and in growth rate is intermediate between the recognized saprophyte *M. phlei* and the slow-growing *M. tuberculosis*. When grown in continuous light on egg-glycerol medium, colonies of these scotochromogens regularly become increasingly reddish orange, and on media such as Proskauer and Beck or 7H9 agar these bacteria are distinctly pigmented. Several possibly applicable species names exist in the literature. Until this taxonomic uncertainty is resolved, it is useful to designate these strains as the *Group II* anonymous ("atypical") mycobacteria.[1]

Data of the Cooperative Survey[2] indicate that *Group II* strains are encountered more commonly than other groups; they occur more commonly in gastric specimens than in sputum. They more commonly than other acid-fast bacilli occur in specimens from patients who have or have had demonstrated infection with tubercle bacilli. *Group II* strains usually occur sporadically, as one or a few colonies, rather than in large numbers, and not consistently from the same patient over periods of weeks to months. (The very few exceptions are analyzed in a recent review.[1]) The conclusion indicated by these and other facts previously reported is that *Group II* strains do not occur as independent pulmonary pathogens. Scotochromogens, however, are the only organisms found in some cases of cervical lymphadenitis.

Another important finding of the survey has been that *Group II* scotochromogens are found also in specimens from patients *not* having pulmonary disease. In fact, about three-fourths of the culture bank *Group II* specimens are from persons not having tuberculosis or other known pulmonary disease. The proportion is not exact. The important point is that no relationship to tuberculosis or to drug treatment is indicated in the occurrence of these strains. Some reports indicate seasonal incidence or evidence of origin in food. A number of occurrences in water are documented. When such contaminated water has been used in the preparation of solutions, colonies have appeared at random in all kinds of specimens. When all reagents used in processing specimens for culture of acid-fast bacilli are kept sterile (as by autoclaving), this contamination by scotochromogens is obviated. These chromogens are probably saprophytes which have a lineage just as ancient as that of tubercle bacilli.

The question then arises, why are the scotochromogens thought by some to occur more commonly in specimens from tuberculous patients who have become sputum-negative for tubercle bacilli? The reliability of this association of scotochromogens with post-chemotherapy resolving tuberculous disease may be doubted; data of the survey as mentioned above, while admittedly indefinite, do not sustain it. It is possible that this supposed relationship appears in part because it is precisely the patients who have become sputum-negative for tubercle bacilli who also fail to raise sputum and hence are followed by gastric lavage testing.

If drug treatment of resolving tuberculous lesions provided conditions favoring the lodgement or emergence of these chromogens, they should be found more commonly in sputum and in resected lung tissue. The record, however, is that *Group II* scotochromogens are relatively uncommon in sputum. Resection is regularly preceded by chemotherapy; but the reported isolation of *Group II* strains from lung tissue is extremely infrequent. If, on the other hand, these chromogens represent contaminants from food or water, rather than inhabitants of pulmonary tissue, they should occur as commonly in gastric specimens from persons lacking evidence of tuberculosis as from patients in sanatoriums. Burrows and Barclay[3] report the finding of only one scotochromogen in gastric specimens from 400 people not having active tuberculosis and never having had antituberculous chemotherapy. This appears to be a low incidence. One might anticipate a higher

[1] Runyon, E. H.: M. Clin. North America, 1959, *43*, 273.

[2] Sponsored jointly by the National Tuberculosis Association and Veterans Administration.

[3] Burrows, W., and Barclay, W. R.: Am. Rev. Tuberc., 1959, *79*, 515.

proportion if the study were continued over a longer period of time, through several seasons; or it may be that this laboratory (University of Chicago Clinics) would have found the same low incidence in specimens from tuberculous or recently tuberculous patients. Further data are needed (a larger series is to be reported by Pratt and Atwell from the State of Ohio Tuberculosis Hospital). The most convincing data will come from a laboratory which makes tests on specimens from both tuberculous and nontuberculous persons.

Burrows and Barclay cite the interesting observations of Tarshis[4] as possibly throwing light on the origin of these scotochromogens. It should be clearly understood that Tarshis' strains, which he thought to have resulted from long-continued drug contact with tubercle bacilli, are not at all comparable with the anonymous ("atypical") mycobacteria. Indeed, it may legitimately be questioned whether Tarshis' organisms are mycobacteria. They are not acid fast.

It may have been supposed that, since scotochromogens are drug resistant, they are favored by chemotherapy which removes other bacilli, just as *Candida spp.* and drug-resistant staphylococci emerge in ever greater numbers following use of broad-spectrum antimicrobials. The parallel, however, does not go beyond the drug resistance. The staphylococci commonly and Candida occasionally are serious pathogens, although they are inhabitants also of normal mucous membranes. No documented case of pulmonary disease due solely to *Group II* scotochromogens is on record. Their usual occurrence as only one or a few colonies, their inconstant appearance in successive specimens, and their quite consistent absence from resected lung tissue suggest no role in disease production. Their frequent appearance apart from drug-treated tuberculosis strongly indicates that their appearance in gastric specimens of patients who have become sputum negative for tuberculosis is fortuitous, or in any event indicates no origin from tubercle bacilli.

[4] TARSHIS, M. S.: Am. Rev. Tuberc., 1958, *78*, 921.

ERNEST H. RUNYON
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May 1, 1959

HISTORIC COLLECTION OF PNEUMOTHORAX MACHINES AND NEEDLES

To the Editor of the American Review of Tuberculosis and Pulmonary Diseases:

There is being formed at the Wellcome Historical Medical Museum a collection of Pneumothorax machines and needles illustrating the various models from the time of the first Forlanini instrument to those invented in the 1940's.

The nucleus of this collection is at present in Leeds and will be shown, before its transfer to the Wellcome Historical Museum, at a meeting of the British Tuberculosis Association and the Yorkshire Thoracic Society in September, 1959. The present collection is nearly complete as regards English models of machines and is complete as regards English needles.

If any of your readers have Continental, British, or American apparatus that is no longer required, I would be grateful for their loan for the Leeds exhibition, or for their gift for the same reason, prior to their transfer to the permanent collection to be kept in London.

THOMAS MARMION
Killingbeck Hospital
York Road
Leeds, 14, England

April 28, 1959

Books

ERGEBNISSE DER TUBERKULOSEFORSCHUNG, Vol. XIV. Edited by St. Engel, L. Heilmeyer, J. Hein, E. Uehlinger. Pp. 735, Gg. Thieme Verlag, Stuttgart, Germany, Price DM 144.

This is volume 14 of the German counterpart of *Advances in Tuberculosis Research*, which is published annually with the aim of making the scientifically interested chest physician acquainted with the latest developments in basic and clinical research. The contributors invited to take part in this endeavor are outstanding men in their fields, and, in order to do justice to the high quality of their papers, the reviewer feels compelled to report briefly on each of their contributions.

The book is most fortunately introduced by a fascinating article by Dr. Engel on the comparative anatomy of the human and mammalian lungs. Using the acinus (in the form conceived by him and the German school, in contrast to Miller's view) as the point of comparison, he describes the pulmonary anatomy of the mouse, rat, guinea pig, bovine, and elephant, and concludes that man displays the relatively biggest acinus. This article, alone, is so well conceived and well written that it makes familiarity with the whole book worth while.

The study of Drs. Töndury and Weibel is concerned with the old problem of the existence and functional significance of arteriovenous anastomoses in the lung. The authors come to the conclusion that mixing of arteriovenous blood can occur only through bronchopulmonary anastomoses. Their study contributes another interesting fact, namely, that formation of longitudinal musculature in "Sperrarterien" is caused by increased pulmonary arterial pressure.

Dr. Köhn, who has in recent years emerged as one of the most outstanding of the younger representatives of German pathology, has contributed a very concise, although detailed, paper on the histologic changes in pulmonary vessels called forth by increased arterial pressure. He has added an extensive bibliography.

The article by Drs. Armstrong and Cudkowicz deals with the pathologic anatomy of the bronchial arteries. The authors emphasize the point that an increase in interstitial tissue is instrumental in increasing the bronchial blood supply, and that insufficiency of the right heart, regardless of cause, furthers the development of anastomoses.

Drs. Esch and Grosse-Brockhoff have devoted their chapter to the role of the heart in tuberculosis. They dwell in great detail on the topics of tuberculous endo-, myo- and epicarditis, and tuberculogenic cor pulmonale. The authors present the reader with highly interesting ideas about the actual extent and significance of shunts in the pulmonary circulation. They discuss and reject Rockitansky's view of the association of pulmonary tuberculosis with valvular deformities.

A most interesting contribution by Drs. Schoenmackers and Vieten correlates roentgenographic with autopsy findings. The authors have filled lungs with contrast media post mortem and compare roentgenograms thus obtained with those taken *in vivo*. Well-chosen case material and excellent illustrations assist in making the paper a valuable source of information for the roentgenologist.

Two Hungarian clinicians, Dr. Görgényi-Göttche and Dr. Kassay, both outstanding authorities in their field, deal with the clinical and physiologic aspects of atelectasis in childhood. In their experience, the child's lung shows an excellent tendency to re-expand even after long-standing atelectasis. They stress the frequency in pertussis of atelectasis which involves most frequently the right middle lobe, the eighth segment, and the lingula. Emphasis is laid upon the significance of tuberculous lymph nodes rupturing into the bronchial tree (one should, perhaps, not forget to mention the work of Schwartz, who has been omitted here, while Hutchinson, Dufourt, and Pepiere have been quoted). The authors deserve acclaim for their illuminating study concerning the differences in anatomic location of the primary tuberculous focus in children and adults. Space prevents a detailed report, but this section is highly recommended to any interested reader in the field.

Dr. Kirchhoff gives a well-written and concise review of the pathology of the mediastinum in childhood which rounds up and extends the preceding article in a most fortunate way.

Dr. Blittersdorf, an authority in public health, gives an excellent and detailed account of the present-day legal situation of the tuberculous patient in Germany. A well-organized system of public supervision and assistance had given way to an almost chaotic situation in the immediate post-war years, which has only recently been remedied by the West German state legislatures, which for a long time failed to fill the medico-legal vacuum left by the collapse of the central German government. Interestingly enough, this situation has been dealt with in the Russian-occupied central part of Germany (the so-called

German Democratic Republic) in a much more efficient manner.

The next article by Drs. Adelberger and Schindler represents one of the few major statistics in the realm of pulmonary surgery published in post-war Germany. The authors' case material comprises 1,306 patients observed over two to seven years. Most of these patients have undergone resection, but the authors believe that pneumothorax must still be considered the method of choice in certain, well-chosen situations.

Dr. Freesen, widely known through his contributions in the field of reticulosis and on the reticuloendothelial system, gives an exceedingly well-written account of the pathologic anatomy and pathogenesis of Boeck's sarcoid. In the limited space of forty pages the author gives a critical and detailed review, which enables the reader to familiarize himself quickly with this highly interesting topic.

With the help of a new and ingenious technique, Drs. Clemens and Villnow have reopened the investigation of pulmonary absorption. They have been able to confirm earlier findings which demonstrated the rapid absorption of distilled water by the alveoli, and have found, in addition, that Na_2HPO_4 is a substance which produces pulmonary edema even in isotonic concentration. Intrabronchial administration of hyaluronidase increases absorption, which is regarded as favoring the existence of a semipermeable mucopolysaccharide alveolar membrane.

The reviewer, who already had the pleasant task of reviewing volume 13 of this series, has little to add to his previous statements, as volume 14 provides the reader in the United States with an opportunity to acquaint himself with the European views in the fields of normal and pathologic pulmonary physiology, two fields which have been rapidly expanding in recent years and which have received many outstanding contributions from German and other European investigative centers.

DIETHELM H. BOEHME
New York, New York

MANUAL OF CHEST CLINIC PRACTICE IN TROPICAL AND SUB-TROPICAL COUNTRIES. By A. J. Benatt. Pp. 100, E. and S. Livingstone Ltd, Edinburgh and London, 1959.

The physician who has had no previous training in chest disease diagnosis or treatment sometimes finds himself assigned today to the job of organizing a chest clinic and providing for the treatment of tuberculous patients. This is not at all an uncommon occurrence in economically underdeveloped countries where the high prevalence of tuberculosis and the expanding ambulatory or domiciliary tuberculosis chemotherapy programs may exhaust the meager supply of chest specialists. In such circumstances this manual would be a most useful guide, for it supplies minute but practical details on the organization of a chest clinic, and information concerning the prevention, diagnosis, and treatment of tuberculosis. Additional information is included on the management of the tuberculous diabetic and the tuberculous pregnant woman and newborn child.

Simple instructions concerning the collection of sputum, laboratory procedures on sputum and urine specimens, techniques for tuberculin testing and BCG vaccination, and care of equipment are presented in a manner that would preclude any misunderstanding. For the physician uninitiated in the management of a tuberculosis treatment program these items of information would be most helpful.

This book is not intended for the student of tuberculosis or for the experienced chest physician. It can be recommended, however, as a very practical handbook for the physician who must undertake the responsibilities of a chest clinic practice despite the lack of formal training in this field.

KURT W. DEUSCHLE
New York, New York

BOOKS RECEIVED

TUBERCULOSIS MEDICAL RESEARCH, NATIONAL TUBERCULOSIS ASSOCIATION, 1904 to 1955. By Virginia Cameron and Esmond R. Long. Pp. 325, National Tuberculosis Association, New York, New York, 1959. Price, $5.00.

CIBA FOUNDATION SYMPOSIUM: BIOSYNTHESIS OF TERPENES AND STEROLS. Edited by G. E. W. Wolstenholme and Cecilia M. O'Connor. Pp. 311, Little, Brown and Company, Boston, Massachusetts, 1959. Price, $8.75.

DIFFICULT DIAGNOSIS: A GUIDE TO THE INTERPRETATION OF OBSCURE ILLNESS. By H. J. Roberts. Pp. 913, W. B. Saunders Company, Philadelphia and London, 1958.

TREATMENT OF LUNG CAVITIES AND ENDOBRONCHIAL TUBERCULOSIS. By Beryl E. Barsby.

Pp. 147, E. &. S. Livingstone Ltd., Edinburgh and London, 1959. Price, $4.75.

DIE TUBERKULOSE: IHRE ERKENNUNG UND BEHANDLUNG. Edited by Hellmuth Deist and Hermann Krauss. Pp. 847, Ferdinand Enke Verlag, Stuttgart W, Hasenbergsteige 3, Germany, 1959.

MIRAGE OF HEALTH. UTOPIAS, PROGRESS, AND BIOLOGICAL CHANGE. By René Dubos. Pp. 236, Harper & Brothers, New York. Price, $4.00.

American Trudeau Society

Medical Section of the National Tuberculosis Association

NOTICES

The following dates and locations of the next three annual meetings of the National Tuberculosis Association and the American Trudeau Society have been selected and approved:

1960—Los Angeles, California—May 15-20
1961—Cincinnati, Ohio—May 21-26
1962—Miami Beach, Florida—May 20-25

ANNUAL MEETING OF THE AMERICAN TRUDEAU SOCIETY

The Medical Sessions Committee *INVITES SUBMISSION OF PAPERS* on all scientific aspects of tuberculosis and nontuberculous respiratory and cardiopulmonary diseases for presentation at the 1960 ANNUAL MEETING IN LOS ANGELES, CALIFORNIA, May 16–18.

Abstracts

1. Abstracts of papers to be offered for presentation should be submitted before January 7, 1960 to the Chairman of the Medical Sessions Committee. EIGHT COPIES are required for distribution to committee members.

2. Abstracts should be limited to a 300-word summary including IMPORTANT DATA AND CONCLUSIONS. Charts and tables should be attached to aid in the selection of papers. Abstracts selected for presentation at the Annual Meeting will be published in THE AMERICAN REVIEW OF RESPIRATORY DISEASES.

3. Reports of interesting or unusual cases will be considered for inclusion in case conferences.

4. The names, academic degrees, titles, and complete addresses of the senior author and coauthors should be included in each abstract.

5. All abstracts will be reviewed by the Medical Sessions Committee. Selection will be largely based on the areas of interest suggested by you and others receiving this invitation.

6. Authors will be notified by February 15, 1960, if their papers have been accepted for presentation.

7. Papers presented at the Annual Meeting must be ORIGINAL contributions NOT PREVIOUSLY PRESENTED OR PUBLISHED. Medical papers presented at the meeting may be submitted to THE AMERICAN REVIEW OF RESPIRATORY DISEASES by the author if he so desires.

Suggestions

The Medical Sessions Committee is particularly anxious to build the 1960 program around your special interests. A memorandum suggesting subjects and speakers would be welcome and should be sent to the chairman by October 15, 1959.

David W. Cugell, M.D., *Chairman*
Medical Sessions Committee
American Trudeau Society
1790 Broadway, New York 19, N. Y.

ATS MEMBERSHIP RULES AMENDED

The American Trudeau Society has amended its bylaws to broaden its base of membership to admit individuals who may not have an M.D. degree and whose training may be in scientific fields not strictly related to medicine. This action is in line with the expanding program of the Society, which now includes all respiratory diseases.

Whereas the requirement for active membership was formerly "a medical degree," with a limited number of Ph.D.'s admitted, the section of the bylaws on membership (Article III) has been amended to read "Active members shall have an M.D., Ph.D., or equivalent degree." At the discretion of the ATS Council, the requirement of a doctoral degree may be waived for a limited number of workers in scientific fields closely related to the activities of the Society.

The bylaws were amended at the annual business meeting of the Society in Chicago on May 26, 1959.

SYMPOSIUM ON EVALUATION OF EARLY DIAGNOSIS OF CANCER

A Symposium on the Evaluation of Early Diagnosis of Cancer will be held at the Annual Scientific Session of the American Cancer Society, to be held October 26–27, 1959, at the Biltmore Hotel, New York, New York.

For further information, write to Dr. Scott Hill, Director, Professional Education, American Cancer Society, Inc., 521 West 57th Street, New York 19, New York.

FOURTH INTERNATIONAL COURSE ON TOMOGRAPHY

The Fourth International Course on Tomography will be held in Genoa, Italy, September 28–October 3, 1959. For further information, write the Secretary General of the Course, Prof. Neopolo Macarini, Istituto di Radiologia, Ospedale San Martino, Genova, Italia.